Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane

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Keywords: Colistin / polymyxin / *Pseudomonas* / *E. coli* / *Klebsiella* / lipopolysaccharide / resistance / MCR-1

Running title: Colistin mechanism of action

Summary

Colistin is an antibiotic of last resort for infections caused by drug-resistant Gram-negative pathogens such as *Pseudomonas aeruginosa* and *Escherichia coli*. For this reason, high rates of treatment failure and resistance to this antibiotic are very concerning, and attempts to resolve these issues are hampered by a poor understanding of colistin's mode of action. Whilst it is well established that colistin binds to lipopolysaccharide in the bacterial outer membrane, it was unclear how this led to bacterial killing. Here, we show that colistin also targets lipopolysaccharide in the cytoplasmic membrane and that this interaction is essential for cytoplasmic membrane permeabilisation, colistin-induced cell lysis and the bactericidal activity of the antibiotic. We also found that MCR-1-mediated colistin resistance confers protection against the antibiotic via the presence of modified lipopolysaccharide within the cytoplasmic membrane, rather than the outer membrane. These findings reveal key details about the mechanism by which colistin kills bacteria, providing the foundations for the development of new approaches to enhance therapeutic outcomes.

62 **Introduction**

63 The emergence of multi-drug-resistant Gram-negative pathogens such as *Pseudomonas aeruginosa*,
64 *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* has necessitated the use of polymyxin
65 antibiotics, which are often the only viable last-resort therapeutic option [1-3].

66 Two closely related polymyxin antibiotics are used clinically, colistin (polymyxin E) and polymyxin B, which
67 share a high degree of structural similarity, consisting of a cationic peptide ring of 7 amino acids connected
68 to a hydrophobic acyl tail by a linear chain of 3 amino acids [1,4]. Polymyxins target lipopolysaccharide (LPS)
69 in the outer membrane (OM) of Gram-negative bacteria via a two-step process (Supplementary Fig. 1). Firstly,
70 the positively-charged peptide ring binds to the anionic lipid A moiety of LPS, resulting in the disruption of
71 cation bridges that stabilise the outer leaflet of the OM. Secondly, the acyl tail of the antibiotic associates
72 with the fatty acid tails of lipid A, causing further OM disruption [1].

73 Polymyxins are rapidly bactericidal towards Gram-negative bacteria *in vitro* but are considerably less
74 efficacious *in vivo*, with up to 70% of patients infected by Gram-negative pathogens failing to respond to
75 colistin treatment [5-8]. In addition to this high burden of treatment failure, a growing threat is the
76 emergence of colistin resistance, which typically arises via chromosomal mutations or the acquisition of
77 mobilized colistin resistance (*mcr*) genes [9]. Chromosomally-mediated colistin resistance can occur via
78 several different pathways, most of which result in the addition of cationic moieties to the normally
79 negatively-charged lipid A. This is thought to result in electrostatic repulsion of the positively-charged
80 polymyxins away from the bacterial surface [10]. By contrast, MCR-mediated resistance is acquired via a
81 plasmid-borne copy of an *mcr* gene, of which 9 homologues have been described to date, following the first
82 report of *mcr*-1 in late 2015 [11,12]. The presence of *mcr* genes on plasmids has become a major cause for
83 concern, with reports of MCR-mediated resistance occurring in several important pathogens including *E. coli*,
84 *Salmonella enterica* and *K. pneumoniae* [13,14]. MCR-1 is thought to be the most prevalent MCR enzyme,
85 having been identified in bacteria isolated from humans, livestock and the environment in Asia, Africa,
86 Europe and North America [14]. Colistin resistance is conferred by the phosphoethanolamine transferase
87 activity of MCR-1, which adds a cationic phosphoethanolamine moiety to the anionic lipid A domain of LPS,
88 reducing its net negative charge and thus decreasing its affinity for the polymyxin antibiotic [11,14].

89 Whilst most colistin resistance occurs via LPS modifications, an alternative mechanism has been observed in
90 *A. baumannii*, which involves the total loss of LPS from the OM [15]. However, this appears to be species
91 specific since other major pathogens such as *P. aeruginosa*, *E. coli* and *K. pneumoniae* cannot tolerate the
92 absence of LPS from the OM [16].

93 Despite the growing importance of polymyxin antibiotics, there are still significant gaps in our understanding
94 of their mode of action. Although it is established that the binding of polymyxins to LPS on the surface of
95 Gram-negative bacteria leads to disruption of the OM, it is not known how this results in cell lysis and

96 bacterial death (Supplementary Fig. 1) [3,17]. It is hypothesised that damage to the LPS monolayer enables
97 polymyxins to traverse the OM via a process of 'self-directed uptake', although this has not been
98 demonstrated experimentally [17,18]. Once across the OM, polymyxins permeabilise the cytoplasmic
99 membrane (CM), which is believed to culminate in bacterial lysis and killing [1-3]. However, the mechanism
100 by which colistin damages the CM is unclear [17]. It has been proposed that the surfactant activity of
101 polymyxins is sufficient to compromise the phospholipid bilayer of the CM via a detergent-like effect [1,2].
102 However, several lines of evidence call into question the ability of colistin to disrupt phospholipid bilayers at
103 physiologically relevant concentrations of the antibiotic. Firstly, as described above, colistin is inactive against
104 LPS-defective *A. baumannii* which has an OM composed of a phospholipid bilayer [15]. Secondly, polymyxins
105 have no activity against Gram-positive bacteria with a CM that consists of a phospholipid bilayer [19]. Finally,
106 studies with model membranes have reported very little disruption to phospholipid bilayers by colistin unless
107 LPS is present [20]. Together, these observations indicate that colistin-mediated disruption of the CM is not
108 due to the engagement of the antibiotic with phospholipids, suggesting that there is an alternative
109 mechanism for how polymyxins damage the CM.

110 LPS is synthesised in the cytoplasm by the Lpx family of enzymes, before it is introduced into the inner leaflet
111 of the CM [21]. It is then flipped to the outer leaflet of the CM by MsbA before transportation to the OM via
112 the LptABCDEFG machinery [22-24]. Therefore, LPS is present in the CM in relatively large quantities, en route
113 to the OM [22-24]. Given the high affinity of polymyxins for LPS, and the well characterised disruption to the
114 LPS-containing OM mediated by these antibiotics, we hypothesised that the presence of LPS in the CM
115 provides a target for polymyxin antibiotics, enabling them to trigger bacterial lysis and death.

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127 **Results**

128 **Colistin targets LPS in the cytoplasmic membrane**

129 Using fluorescent BoDipy-labelled polymyxin B nonapeptide (PMBN) as a probe for LPS at the cell surface, we
130 were able to detect LPS in the OM of the model Gram-negative pathogen *P. aeruginosa* PA14 (Fig. 1a,
131 Supplementary Fig. 2) [25]. By producing spheroplasts of *P. aeruginosa* cells, we also found that BoDipy-
132 PMBN bound to the CM, demonstrating that LPS was present in the CM in relatively large quantities (Fig. 1b,
133 Supplementary Fig. 3). To investigate if LPS in the CM was a target for polymyxin antibiotics, we first
134 attempted to reduce the amount of LPS in the CM by blocking LPS biosynthesis with either the LpxC inhibitor
135 CHIR090, or the fatty acid biosynthesis inhibitor cerulenin [16,26]. Exposure of *P. aeruginosa* to sub-lethal
136 concentrations of CHIR090 or cerulenin resulted in almost complete depletion of LPS from the CM, with no
137 significant effects on LPS levels in the OM and only a minimal effect on the growth rate of bacteria (Fig. 1abcd,
138 Supplementary Fig. 4).

139 To test whether CHIR090 or cerulenin had any effect on the interaction of colistin or polymyxin B with LPS in
140 the OM of *P. aeruginosa* cells, we used the *N*-phenyl-1-napthylamine (NPN) fluorophore to measure OM
141 permeablisation caused by the polymyxin antibiotics [27]. This revealed that both colistin and polymyxin B
142 caused immediate and equal disruption of the OM of untreated bacteria and those exposed to CHIR090 or
143 cerulenin, confirming that the LPS biosynthesis inhibitors did not modulate the levels of LPS in the OM (Fig.
144 1ef, Supplementary Fig. 5).

145 Having established that depletion of LPS from the CM using CHIR090 or cerulenin had no effect on the
146 interaction of colistin or polymyxin B with the OM, we turned our attention to the interaction of the
147 polymyxin antibiotics with the CM. To do this, we measured the binding of BoDipy-labelled colistin or BoDipy-
148 polymyxin B to *P. aeruginosa* cells exposed, or not, to CHIR090 or cerulenin (Supplementary Fig. 6, 7). In the
149 absence of the inhibitors of LPS biosynthesis, there was a rapid and high level of binding of the labelled
150 polymyxin antibiotics to bacteria (Fig. 1gh). By contrast, when LPS was depleted from the CM using CHIR090
151 or cerulenin, the binding of BoDipy-colistin or BoDipy-polymyxin B was significantly reduced, indicating that
152 LPS in the CM was targeted by the polymyxin antibiotics (Fig. 1gh).

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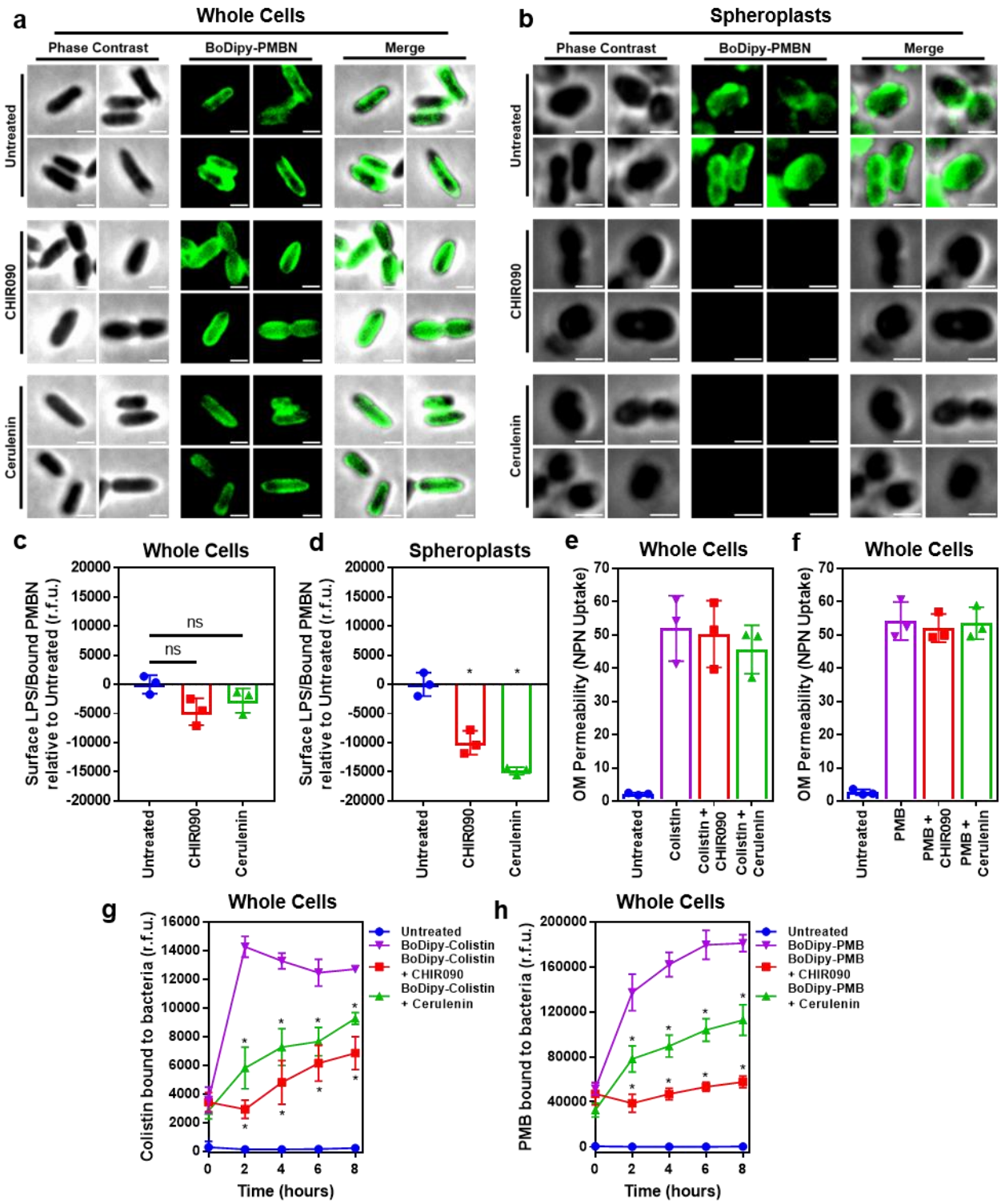

Figure 1. Depletion of LPS from the cytoplasmic membrane of *P. aeruginosa* reduces colistin binding to cells. a, b, Representative fluorescence microscopy images visualising LPS in the OM of *P. aeruginosa* PA14 whole cells **(a)** or in the CM of spheroplasts of *P. aeruginosa* **(b)** in the absence of treatment, or after exposure to the LPS biosynthesis inhibitors CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) for 2 hours, using 7 µg ml^{-1} BoDipy-polymyxin B nonapeptide (PMBN) (Scale bars: 5 µm). **c, d,** Quantification of amount of LPS in the OM of *P. aeruginosa* PA14 whole cells **(c)** or in the CM of *P. aeruginosa* spheroplasts **(d)** exposed, or not, to CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) for 2 hours, as determined by fluorescence of BoDipy-PMBN (7 µg ml^{-1}) bound to the cell surface (n=3 in triplicate; ns: p>0.05, *p<0.001

167 compared to Untreated conditions). **e, f,** OM disruption of *P. aeruginosa* PA14 cells during 10 minutes of exposure to
168 colistin (4 µg ml^{-1}) **(e)** or polymyxin B (PMB, 4 µg ml^{-1}) **(f)**, in the absence or presence of CHIR090 (0.125 µg ml^{-1}) or
169 cerulenin (32 µg ml^{-1}), as assessed by uptake of the fluorescent dye NPN (10 µM) (n=3, each data point represents the
170 arithmetic mean of 20 replicate measurements; no significant differences (p>0.05) between polymyxin treated
171 bacteria). **g, h,** Binding of fluorescent BoDipy-labelled colistin (5 µg ml^{-1}) **(g)** or BoDipy-PMB (10 µg ml^{-1}) **(h)** to *P.*
172 *aeruginosa* PA14 cells in the absence or presence of CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) (n=4; *p<0.0001
173 compared to BoDipy-labelled antibiotics alone). Data in **c, d** were analysed by a one-way ANOVA with Dunnett's post-
174 hoc test. Data in **e, f** were analysed by a one-way ANOVA with Tukey's post-hoc test. Data in **g, h** were analysed by a
175 two-way ANOVA with Dunnett's post-hoc test. Data are presented as the arithmetic mean, and error bars represent the
176 standard deviation of the mean. OM: outer membrane; CM: cytoplasmic membrane; NPN: *N*-phenyl-1-napthylamine;
177 r.f.u.: relative fluorescence units.

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179 **The bactericidal activity of colistin requires the presence of LPS in the cytoplasmic membrane**

180 Having demonstrated that colistin and polymyxin B target LPS in the CM, we wanted to understand whether
181 this interaction was needed for the bactericidal activity of the antibiotics. To do this, we depleted LPS from
182 the CM of *P. aeruginosa* using CHIR090 or cerulenin and measured bacterial survival over time during
183 exposure to the polymyxins. As expected, colistin alone caused a rapid and substantial drop in c.f.u. counts
184 of bacteria in the absence of inhibitors of LPS biosynthesis, with >10,000-fold reduction in survival within 4
185 hours (Fig. 2ab). By contrast, there was almost no decrease in c.f.u. counts over 8 hours when *P. aeruginosa*
186 was exposed to colistin in the presence of CHIR090 or cerulenin to deplete LPS from the CM (Fig. 2ab). This
187 effect was dose-dependent, with the protection conferred by CHIR090 and cerulenin against colistin-
188 mediated killing reduced as the concentration of the LPS biosynthesis inhibitors was decreased
189 (Supplementary Fig. 8). Removing LPS from the CM with CHIR090 or cerulenin also prevented polymyxin B
190 from exerting its bactericidal effects, with >1000-fold increased survival of *P. aeruginosa* in the presence of
191 the inhibitors of LPS biosynthesis compared to bacteria exposed to polymyxin B alone (Fig. 2c). Furthermore,
192 the protective effects of CHIR090 and cerulenin could also be observed even after bacteria were first exposed
193 to colistin. After 2 hours incubation with colistin, c.f.u. counts of *P. aeruginosa* dropped approximately 500-
194 fold compared to the starting inoculum (Fig. 2d). In the absence of CHIR090 or cerulenin, killing continued
195 with an additional 1000-fold decrease in survival over the next 4 hours (Fig. 2d). However, the addition of
196 cerulenin after 2 hours completely halted bacterial killing, whilst the introduction of CHIR090 reduced the
197 rate of killing significantly (Fig. 2d). Therefore, depletion of LPS from the CM significantly inhibited the
198 bactericidal activity of colistin against *P. aeruginosa*, even after the antibiotic had bound to cells.

199 Since perturbation of LPS biosynthesis by CHIR090 or cerulenin may have triggered stress responses
200 activating the PmrAB or PhoPQ two-component systems that modulate the susceptibility of *P. aeruginosa* to
201 colistin, we repeated the killing experiments using transposon mutants with disrupted copies of *pmrA*, *pmrB*,

202 *phoP* or *phoQ* [28,29]. As expected from previous work, the *phoQ*-disrupted mutant was less susceptible to
203 colistin than the WT strain (Supplementary Fig. 9) [30]. However, the presence of CHIR090 or cerulenin
204 significantly reduced the killing of all four mutants by colistin, indicating that depletion of LPS from the CM,
205 rather than the induction of stress responses and modification of LPS in the OM, was responsible for the
206 protection conferred by the LPS biosynthesis inhibitors against colistin (Supplementary Fig. 9). Moreover,
207 neither CHIR090 nor cerulenin had any effect on the ability of the bactericidal agents ciprofloxacin,
208 gentamicin, imipenem or ethylenediaminetetraacetic acid (EDTA) to kill *P. aeruginosa*, further confirming
209 that the effects of blocking LPS biosynthesis were specific to polymyxins, and not a consequence of general
210 stress responses reducing antibiotic susceptibility (Supplementary Fig. 10).

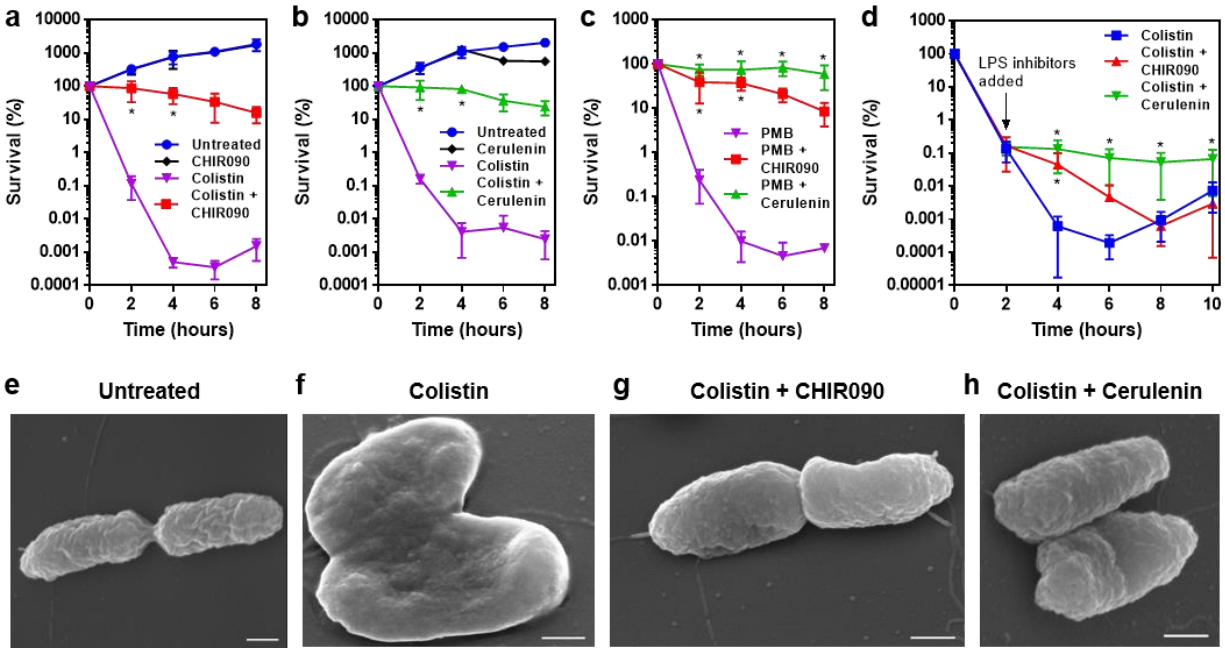
211 Since growth rate can affect the bactericidal activity of antibiotics, it was possible that the protection
212 provided by CHIR090 or cerulenin against colistin-mediated killing was due to their slight inhibitory effects
213 on cell replication (Supplementary Fig. 4) [31,32]. However, a range of completely growth-inhibitory
214 concentrations of the bacteriostatic antibiotics tetracycline or chloramphenicol had almost no effect on
215 colistin-mediated killing, confirming that alterations in growth rate due to the presence of CHIR090 or
216 cerulenin were not the basis of their protective effects against polymyxins (Supplementary Fig. 11).
217 Additionally, we confirmed using checkerboard broth microdilution assays that neither LPS biosynthesis
218 inhibitor displayed synergy with colistin, suggesting that CHIR090 and cerulenin did not have enhanced
219 growth-inhibitory effects in combination with the polymyxin antibiotic (Supplementary Fig. 12). This also
220 revealed that the inhibitors of LPS biosynthesis did not directly interfere with colistin, for example by binding
221 to the polymyxin (Supplementary Fig. 12).

222 After isolating *P. aeruginosa* mutants that were resistant to CHIR090, we found that the LPS biosynthesis
223 inhibitor did not prevent colistin-mediated killing of these CHIR090-resistant strains, demonstrating that the
224 inhibitor was not blocking the bactericidal effects of the polymyxin antibiotic via off-target effects
225 (Supplementary Fig. 13). Furthermore, we tested whether either CHIR090 or cerulenin triggered *P.*
226 *aeruginosa* to release outer membrane vesicles (OMVs) that could inactivate colistin extracellularly by
227 measuring the concentration of membrane phospholipids and LPS in the culture supernatant [33]. Exposure
228 of bacteria to the LPS biosynthesis inhibitors in combination with colistin resulted in a decrease in the amount
229 of these key OMV components shed from the cell surface, with higher levels of colistin activity maintained in
230 the supernatant in the presence of CHIR090 or cerulenin (Supplementary Fig. 14, 15). From these
231 experiments, we concluded that the protection conferred by the inhibitors of LPS biosynthesis against killing
232 of *P. aeruginosa* by polymyxins was specifically due to their effects on depleting LPS from the CM.

233 To determine whether the protective effects of CHIR090 or cerulenin also applied to other Gram-negative
234 pathogens, we exposed clinical isolates of *E. coli* and *K. pneumoniae*, as well as *P. aeruginosa*, to colistin in
235 the absence or presence of sub-lethal concentrations of the LPS biosynthesis inhibitors. As observed for *P.*
236 *aeruginosa* PA14, there was a large and rapid drop in c.f.u. counts of all strains exposed to colistin alone, but

237 the introduction of either CHIR090 or cerulenin significantly reduced the rate and magnitude of killing,
238 suggesting the polymyxin antibiotic had a broadly conserved mechanism against Gram-negative bacteria
239 (Supplementary Fig. 16). Next, we used scanning electron microscopy (SEM) to examine the morphology of
240 *P. aeruginosa* cells exposed to colistin alongside either CHIR090 or cerulenin. As expected, in the absence of
241 antibiotics, bacteria had a typical rod-like appearance, whilst cells exposed to colistin had a greatly altered
242 morphology, with a distorted and deflated appearance (Fig. 2ef). However, cells exposed to colistin in the
243 presence of CHIR090 or cerulenin had an almost normal appearance, underlining the protective effects of
244 LPS depletion from the CM (Fig. 2gh, Supplementary Fig. 17).

245 In summary, these findings revealed that the bactericidal activity of polymyxin antibiotics was dependent
246 upon the presence of LPS in the CM of clinically-important pathogens.



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248 **Figure 2. Depletion of LPS from the cytoplasmic membrane protects *P. aeruginosa* from colistin. a,** Survival of *P.*
249 *aeruginosa* PA14 cells exposed to colistin (4 µg ml^{-1}), the LPS biosynthesis inhibitor CHIR090 (0.125 µg ml^{-1}), both
250 antibiotics, or neither (n=4; *p<0.01 compared to Colistin alone). Values for CHIR090 alone and Untreated overlap. **b,**
251 Survival of *P. aeruginosa* PA14 cells during exposure to colistin (4 µg ml^{-1}), the LPS biosynthesis inhibitor cerulenin (32
252 µg ml^{-1}), both antibiotics, or neither (n=4; *p<0.0001 compared to Colistin alone). **c,** Survival of *P. aeruginosa* PA14 cells
253 during exposure to polymyxin B (PMB, 4 µg ml^{-1}) alone, or in combination with CHIR090 (0.125 µg ml^{-1}) or cerulenin (32
254 µg ml^{-1}) (n=4; *p<0.05 compared to PMB alone). **d,** Survival of *P. aeruginosa* PA14 cells exposed to colistin (4 µg ml^{-1})
255 alone for 2 hours, followed by no further treatment, or the addition of either CHIR090 (0.125 µg ml^{-1}) or cerulenin (32
256 µg ml^{-1}) (n=3; *p<0.05 compared to Colistin alone). **e-h,** Representative scanning electron microscopy (SEM) images of
257 *P. aeruginosa* PA14 cells incubated in broth only **(e)**, broth containing colistin (4 µg ml^{-1}) alone **(f)**, colistin (4 µg ml^{-1}) and
258 CHIR090 (0.125 µg ml^{-1}) **(g)**, or colistin (4 µg ml^{-1}) and cerulenin (32 µg ml^{-1}) **(h)**, for 2 hours in each case (Scale bars: 200
259 nm). Data in **a-d** were analysed by a two-way ANOVA with Sidak's **(a, b)**, Dunnett's **(c)** or Holm-Sidak's **(d)** post-hoc tests.
260 Data are presented as the arithmetic mean, and error bars represent the standard deviation of the mean.

261 **Colistin-mediated lysis is dependent on the presence of LPS in the cytoplasmic membrane**

262 Having established that the bactericidal activity of colistin required the presence of LPS in the CM, we wanted
263 to determine whether this was due to colistin-mediated CM damage being dependent on interactions with
264 LPS in the CM. To do this, we first examined how variations in the amount of LPS in the CM of whole *P.*
265 *aeruginosa* cells affected permeabilisation of the CM by polymyxin antibiotics. As expected, exposure of
266 bacteria to colistin or polymyxin B alone caused disruption of the CM, resulting in a significant signal from
267 the DNA-reactive fluorophore propidium iodide (PI) (Fig. 3ab, Supplementary Fig. 18) [34]. By contrast, cells
268 exposed to colistin when LPS was depleted from the CM with the LPS biosynthesis inhibitors CHIR090 or
269 cerulenin had an almost absent PI signal, indicating that the CM remained intact (Fig. 3ab). Similar results
270 were seen with clinical isolates of *P. aeruginosa*, *K. pneumoniae* and *E. coli,* with CHIR090 significantly
271 reducing colistin-mediated permeabilisation of the CM (Supplementary Fig. 19).

272 To confirm that these data arose from the lack of LPS in the CM, we produced spheroplasts enclosed by only
273 a CM from *P. aeruginosa* cells that had been exposed, or not, to CHIR090 or cerulenin. In the absence of any
274 antibiotics, there was a gradual loss of integrity of the spheroplast CM, as determined by the signal from PI
275 staining (Fig. 3c). By contrast, challenging the spheroplasts with colistin resulted in a high level of
276 fluorescence, indicative of severe CM permeabilisation (Fig. 3c). However, when spheroplasts from bacteria
277 pre-treated with CHIR090 or cerulenin to deplete LPS from the CM were exposed to colistin, the signal from
278 PI was similar to that seen in the absence of the polymyxin antibiotic (Fig. 3c). Therefore, the ability of colistin
279 to disrupt the CM of *P. aeruginosa* was dependent upon the presence of LPS in the CM.

280 To test whether this requirement of LPS in the CM for polymyxin-mediated CM disruption was also associated
281 with bacterial lysis, we measured the optical density (OD_{595nm}) of bacterial suspensions exposed to colistin or
282 polymyxin B in the absence or presence of the LPS biosynthesis inhibitors CHIR090 or cerulenin. Whilst
283 colistin alone caused lysis of *P. aeruginosa*, with a steady decrease in OD_{595nm} readings observed, this was
284 completely blocked in the presence of CHIR090 or cerulenin (Fig. 3d). Almost identical data were produced
285 when bacteria were challenged with polymyxin B, or with *P. aeruginosa* spheroplasts exposed to colistin (Fig.
286 3ef). In all cases, when the CM was depleted of LPS using the inhibitors of LPS biosynthesis, there was no
287 detectable cell lysis triggered by the polymyxin antibiotics (Fig. 3def). This finding was then extended to
288 clinical isolates of Gram-negative pathogens, with CHIR090 significantly reducing colistin-mediated lysis in *P.*
289 *aeruginosa*, *E. coli* and *K. pneumoniae* strains (Supplementary Fig. 20).

290 Taken together, these data demonstrate that polymyxins were dependent on the presence of LPS in the CM
291 in order to permeabilise the CM and cause bacterial lysis.

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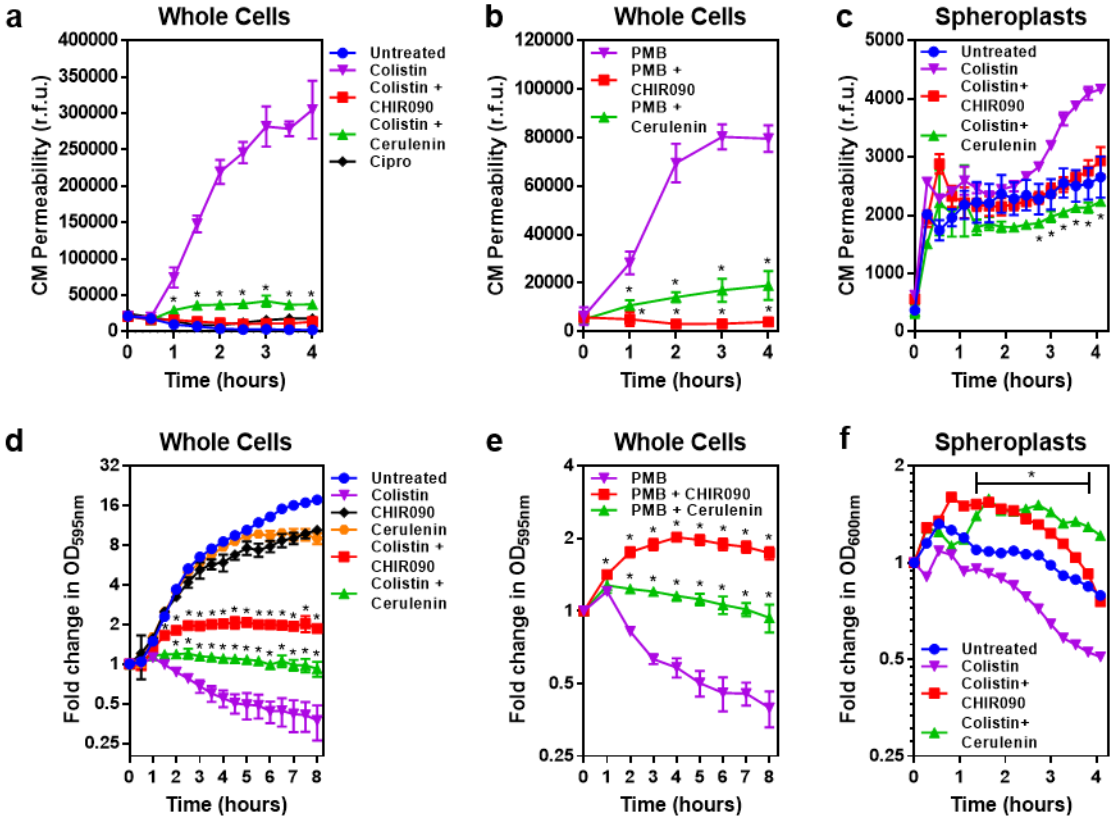

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295 **Figure 3. Depletion of LPS from the cytoplasmic membrane prevents colistin-mediated lysis of *P. aeruginosa*. a,**
296 Disruption of the CM of *P. aeruginosa* PA14 cells during incubation with colistin (4 µg ml^{-1}) in the absence or presence
297 of CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), or with a bactericidal concentration of ciprofloxacin (4 µg ml^{-1}) as a
298 negative control, as determined using 2.5 µM propidium iodide (PI) (n=3 in duplicate; *p<0.0001 compared to Colistin
299 alone for all populations below the asterisks). **b,** CM disruption of *P. aeruginosa* PA14 cells exposed to polymyxin B
300 (PMB, 4 µg ml^{-1}) in the absence or presence of CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), as determined using
301 2.5 µM PI (n=4; *p<0.0001 compared to PMB alone). **c,** Permeabilisation of the CM of *P. aeruginosa* PA14 spheroplasts
302 by colistin (4 µg ml^{-1}) following exposure, or not, of bacteria to CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) for 2
303 hours prior to conversion to spheroplasts, as determined using 0.25 µM PI (n=3, experiment performed on 3
304 independent occasions; *p<0.05 for CHIR090 and cerulenin pre-treated spheroplasts compared to spheroplasts exposed
305 only to colistin). **d, e,** Lysis of *P. aeruginosa* PA14 cells during exposure to colistin (4 µg ml^{-1}) **(d)** or PMB (4 µg ml^{-1}) **(e)**
306 in the absence or presence of CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), as measured using OD_{595nm} readings
307 (n=4; *p<0.05 compared to Colistin/PMB alone). **f,** Lysis of *P. aeruginosa* PA14 spheroplasts in response to colistin (4 µg
308 ml^{-1}) following exposure, or not, of bacteria to CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) for 2 hours prior to
309 conversion to spheroplasts, measured by OD_{600nm} readings (n=3, experiment performed on 3 independent occasions;
310 *p<0.05 for CHIR090 and cerulenin pre-treated spheroplasts compared to spheroplasts exposed only to colistin, error
311 bars are omitted for clarity). Data in **a-f** were analysed by a two-way ANOVA with Dunnett's post-hoc test. Data are
312 presented as the arithmetic mean, and error bars, where shown, represent the standard deviation of the mean. CM:
313 cytoplasmic membrane; r.f.u.: relative fluorescence units; OD: optical density.

314

315 **MCR-1 confers colistin resistance via modified LPS in the cytoplasmic membrane**

316 Colistin resistance is a growing concern, particularly the plasmid-borne mobilized colistin resistance (*mcr*)
317 family of resistance determinants that add a phosphoethanolamine group to the lipid A domain of LPS
318 [10,11]. It has been proposed that the increased positive charge of LPS molecules conferred by the cationic
319 phosphoethanolamine moiety leads to electrostatic repulsion of colistin away from the surface of bacteria
320 [10,11]. However, previous work has shown that colistin permeabilises the OM of bacteria expressing *mcr*-1
321 almost as efficiently as strains that do not express *mcr*-1 [17]. Notably, it has also been demonstrated that
322 MCR-1 is present in the CM, suggesting this is where modifications to LPS occur, before the molecules are
323 transported to the OM [11,35]. Therefore, having established that colistin kills Gram-negative pathogens by
324 targeting LPS in the CM, we hypothesised that MCR-1, which prevents polymyxin-mediated bacterial lysis,
325 confers colistin resistance due to the presence of modified LPS in the CM rather than the OM [17].

326 To investigate this hypothesis, we used an *E. coli* strain, the most common host organism for these resistance
327 genes, expressing *mcr-1* from a plasmid [36]. As expected, *E. coli* expressing *mcr-1* had a significantly greater
328 colistin minimum inhibitory concentration (MIC, 2 µg ml^{-1}) compared to an otherwise isogenic empty plasmid
329 control strain that did not express the resistance determinant (0.25 µg ml^{-1}) (Supplementary Fig. 21). This
330 confirmed that MCR-1 was being produced by the *E. coli* cells and that it was functional, whilst expression of
331 *mcr-1* did not result in a significant growth defect (Supplementary Fig. 21).

332 Next, we verified previous findings by showing that colistin, across a range of antibiotic concentrations,
333 caused similar levels of OM disruption in the *E. coli* strain expressing *mcr-1* and in *E. coli* cells harbouring only
334 an empty plasmid (Fig. 4a) [17]. Thus, it appeared that MCR-1 did not mediate colistin resistance at the level
335 of the OM. By contrast, whilst colistin compromised the CM of control empty plasmid-containing *E. coli*, it
336 had no effect on the CM of bacteria expressing *mcr*-1, as determined by the entry of PI into the cytoplasm
337 (Fig. 4b). This suggested that MCR-1 protected bacteria from colistin-mediated permeabilisation of the CM,
338 not the OM. In keeping with this, colistin induced lysis of *E. coli* with only the empty plasmid, but cells
339 expressing *mcr-1* with an intact CM grew in the presence of the antibiotic (Fig. 4c).

340 To further test whether MCR-1 protected bacteria from colistin by modifying LPS in the CM, we examined
341 the activity of the polymyxin antibiotic against spheroplasts generated from *E. coli* whole cells either
342 expressing *mcr-1* or harbouring an empty plasmid (Supplementary Fig. 22). Colistin caused significant CM
343 damage and triggered cell lysis in empty plasmid-containing *E. coli* spheroplasts (Fig. 4de). However, against
344 spheroplasts of the *E. coli* strain producing MCR-1, the ability of colistin to disrupt the CM was minimal, and
345 there was no observable lysis in response to the polymyxin (Fig. 4de). Therefore, we concluded that MCR-1
346 mediated colistin resistance by modifying LPS in the CM, which prevented the antibiotic from permeabilising
347 the CM and causing bacterial lysis.

348

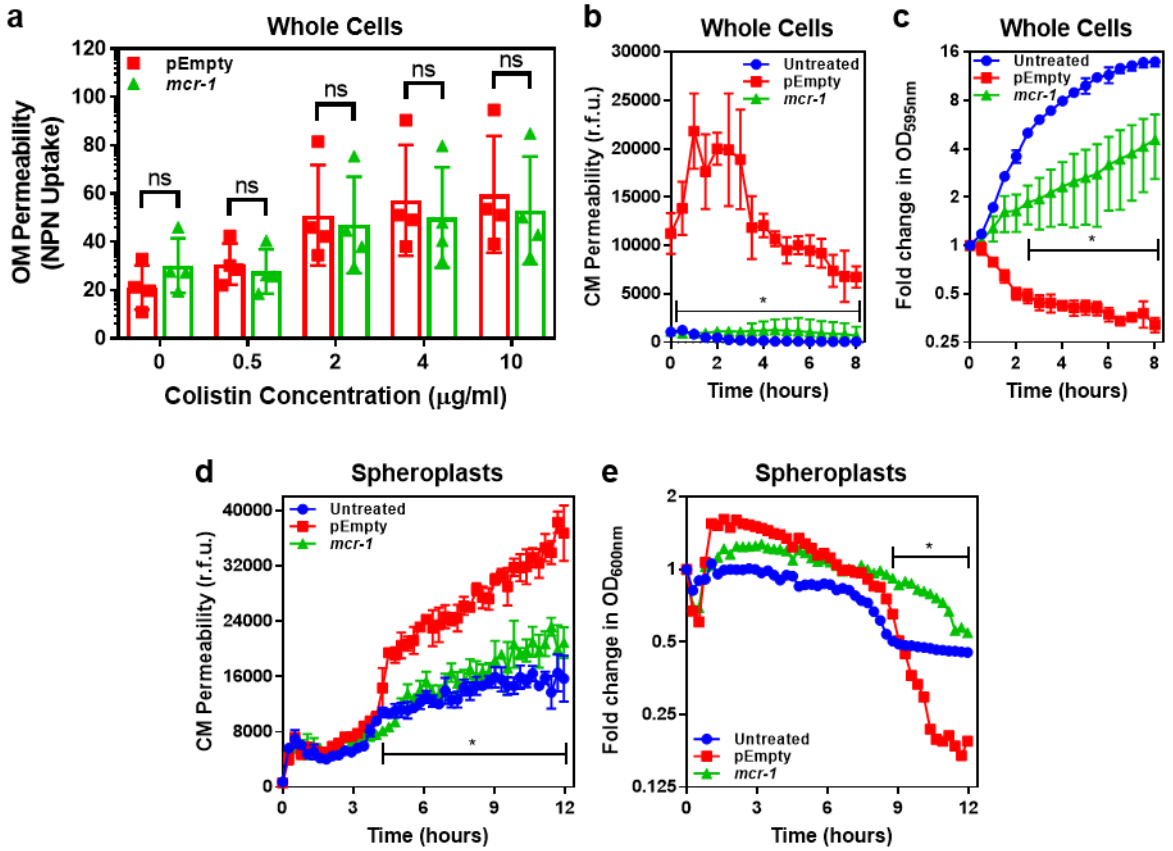
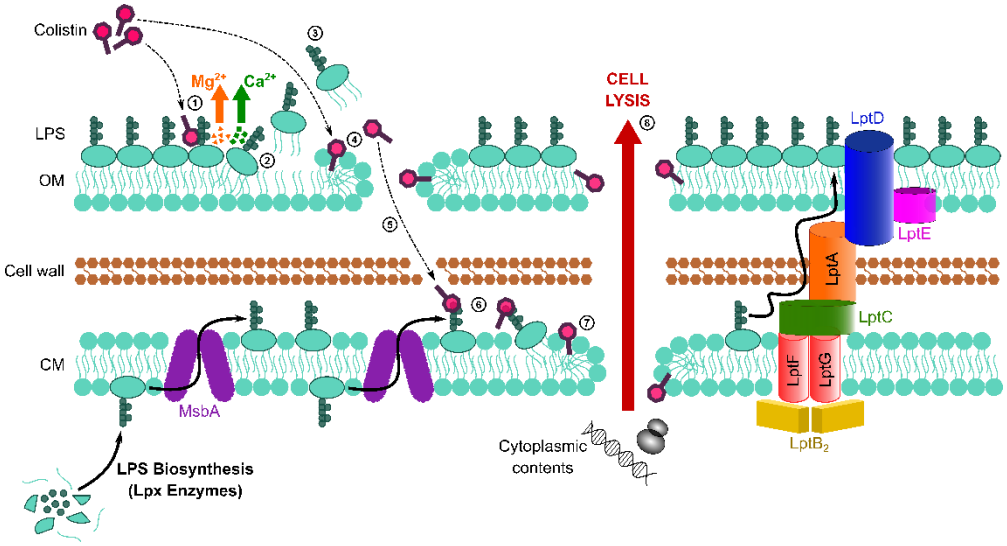


Figure 4. Colistin disrupts the outer membrane but not the cytoplasmic membrane of *E. coli* expressing *mcr*-1. a, OM disruption of *E. coli* MC1000 cells expressing *mcr-1* or an empty plasmid control strain (pEmpty) during 10 minutes of exposure to colistin at the indicated antibiotic concentrations, as determined by uptake of the fluorescent dye NPN (10 µM) (n=4; ns: p>0.05 between pEmpty and *mcr-1* strains). **b,** Permeabilisation of the CM of *E. coli* MC1000 cells expressing *mcr-1* or empty plasmid-containing cells during incubation with colistin (4 µg ml^{-1}), as determined using 2.5 µM propidium iodide (PI) (n=4, each data point represents the arithmetic mean of 20 replicate measurements; ns: p>0.05 between pEmpty and *mcr-1* strains). **c,** Growth or lysis of *E. coli* MC1000 cells expressing *mcr-1* or empty plasmid control cells during exposure to colistin (4 µg ml^{-1}), as measured using OD_{595nm} readings (n=4; *p<0.05 compared to pEmpty). **d,** Permeabilisation of the CM of *E. coli* MC1000 spheroplasts generated from bacteria expressing *mcr-1* or empty plasmid control bacteria during incubation with colistin (4 µg ml^{-1}), as determined using 0.25 µM PI (µM) (n=3, experiment performed on 4 independent occasions; *p<0.0001 compared to pEmpty). **e,** Lysis of *E. coli* MC1000 spheroplasts generated from bacteria expressing *mcr-1* or empty plasmid-containing bacteria during exposure to colistin (4 µg ml^{-1}), as measured using OD_{595nm} readings (n=3, experiment performed on 4 independent occasions; *p<0.01 compared to pEmpty, error bars are omitted for clarity). Data in **a-e** were analysed by a two-way ANOVA with Sidak's **(a)** or Dunnett's **(b-e)** post-hoc tests. Data are presented as the arithmetic mean, and error bars, where shown, represent the standard deviation of the mean. OM: outer membrane; CM: cytoplasmic membrane; NPN: *N*-phenyl-1-napthylamine; r.f.u.: relative fluorescence units; OD: optical density.

369 **Discussion**

370 Colistin is an increasingly important last-resort antibiotic used to combat infections caused by multi-drug-
371 resistant Gram-negative pathogens, including *P. aeruginosa*, *K. pneumoniae* and *E. coli* [1-3]. However,
372 treatment failure occurs frequently, and resistance is a growing concern [5-9]. Efforts to address these issues
373 are compromised by a poor understanding of colistin's bactericidal mode of action. Whilst the initial
374 interactions of colistin with LPS in the OM of Gram-negative bacteria were well-established, it was unclear
375 how the antibiotic traversed the OM and damaged the CM to cause cell lysis (Supplementary Fig. 1). In this
376 work, we demonstrate that colistin targets LPS in the CM prior to transport to the OM, resulting in membrane
377 permeabilization, bacterial lysis and killing (Fig. 5).



378

379 **Figure 5. Colistin kills bacteria by targeting LPS in both the outer and cytoplasmic membranes, leading to disruption**
380 **of the cell envelope and bacterial lysis.** Diagrammatic representation of the novel proposed mechanism of action of
381 colistin: Colistin binds to LPS in the OM (1), displacing cations that form bridges between LPS molecules, which leads to
382 destabilisation of the OM (2). As a consequence of the weakening of intermolecular bonds in the LPS monolayer, LPS is
383 released from the bacterial surface (3), allowing colistin to further damage the OM via the action of the polymyxin lipid
384 tail (4). This provides colistin with access to the periplasm, where colistin interacts with LPS in the CM (5) that is awaiting
385 transport to the OM by the LptABCDEFG machinery after being synthesized in the cytoplasm and flipped to the outer
386 leaflet of the CM by MsbA. As in the OM, colistin binding to LPS results in disruption of the CM (6), which it ultimately
387 permeabilises (7), culminating in the loss of cytoplasmic contents, cell lysis and bacterial death (8).

388 It has previously been hypothesised that polymyxins damage the phospholipid bilayer in the CM via a
389 detergent-like effect due to the surfactant properties of the antibiotic lipid tail [1,2]. However, our data
390 showing that colistin requires LPS to be present in the CM in order to kill bacteria explains how an antibiotic
391 with high affinity and specificity for LPS causes disruption to both the OM and CM. Furthermore, these
392 findings provide support for the observations that colistin does not damage the OM of resistant *A. baumannii*
393 isolates where LPS has been replaced by a phospholipid bilayer, and that polymyxins cause only minimal
394 disruption to model phospholipid membranes unless LPS is present [15,20].

395 Whilst the interaction of colistin with LPS in the CM is likely to share similarities with the same process at the
396 OM, there are also likely to be differences owing to the differing concentrations of LPS between the two
397 membranes [21]. In the OM, LPS is a highly abundant component with molecules tightly packed and stabilised
398 with cation bridges. By contrast, LPS is a minority component in the CM, which may affect the rate and degree
399 to which the CM is disrupted by polymyxins. For example, previous work showed a direct correlation between
400 the concentration of LPS in a model membrane and the extent to which the membrane was permeabilised
401 by colistin [20].

402 In keeping with this, we observed that colistin induced OM damage within minutes of bacterial exposure to
403 the antibiotic, but disruption of the CM took much longer. Part of this delay may be due to the time taken
404 for the polymyxin to traverse the OM, since polymyxins accumulate on the bacterial surface before migrating
405 to the CM [37]. However, when spheroplasts lacking an OM were exposed to colistin, it still took more than
406 2 hours for CM permeabilisation to occur. Therefore, it appears that colistin-mediated disruption of the CM
407 is considerably less efficient than that of the OM.

408 Additional evidence for polymyxin targeting of LPS in the CM came from our studies with *E. coli* expressing
409 *mcr-1*, a plasmid-borne colistin resistance determinant that is the most prevalent member of the *mcr* family
410 of gene homologues and a growing cause for concern. MCR-1 functions by modifying the lipid A domain of
411 LPS with a phosphoethanolamine moiety, preventing colistin binding. However, recent work showed that
412 MCR-1 did not prevent colistin-mediated OM permeabilisation, calling into question how this enzyme confers
413 polymyxin resistance [17]. Phosphoethanolamine modifications of LPS have been shown to occur in the CM
414 before trafficking to the OM, and our data demonstrate that it is the presence of modified LPS in the CM that
415 confers protection from colistin on *E. coli* expressing *mcr-1*, since the CM of spheroplasts from MCR-1-
416 producing bacteria was not damaged by the polymyxin antibiotic [35].

417 All *mcr* homologues are thought to act at the CM, so it is likely that our findings are generalizable to this
418 mechanism of colistin resistance [11-14]. However, colistin resistance in other species that occurs via
419 chromosomal mutations or the induction of stress responses is more complex, and it remains to be seen
420 whether these diverse mechanisms confer colistin protection through modified LPS in the CM or OM [28-
421 30,38]. It is also not yet clear why colistin is able to disrupt the OM but not CM of bacteria expressing *mcr-1*.
422 However, as discussed above, there are likely to be important differences in antibiotic activity due to the
423 relative abundance of modified LPS in the CM versus OM.

424 In summary, this work contributes to our understanding of the mechanism of action of colistin by
425 demonstrating that LPS in the CM is targeted by the polymyxin and is required for the bactericidal and lytic
426 activities of the antibiotic. In addition, our findings provide insight into how *mcr*-1 confers colistin resistance,
427 laying the foundations for developing new therapeutic strategies to enhance the efficacy of colistin.

428

429 ## Methods

430 ### Bacterial strains and growth conditions

431 The bacterial strains used in this study are listed in Supplementary Table 1. For each experiment, bacteria
432 were grown for 18 h to stationary phase at 37°C with shaking (180 r.p.m.). All strains were grown in Luria
433 broth (LB; Thermo Fisher Scientific, USA), with the exception of *P. aeruginosa* strain A23, which was cultured
434 in Tryptic Soy Broth (TSB; BD Biosciences, USA), and *K. pneumoniae* strain KPC, which was grown in Brain
435 Heart Infusion Broth (BHI; VWR International, USA). For routine culture of bacteria on solid media, strains
436 were grown on the relevant media as described above supplemented with 1.5% technical agar (BD
437 Biosciences). Growth media were supplemented with chloramphenicol (25 µg ml^{-1}), gentamicin (15 µg ml^{-1}),
438 tetracycline (12.5 µg ml^{-1}) or 0.5 mM isopropyl-β-D-thiogalactoside (IPTG, Melford Laboratories, UK) where
439 required. Enumeration of bacterial c.f.u. was done by plating 10-fold serial dilutions of bacterial cultures on
440 to Mueller-Hinton agar (MHA; Thermo Fisher Scientific) plates. Inoculated agar plates were incubated
441 statically for 18 h in air at 37°C.

442 ### Determination of minimum inhibitory concentrations of antibiotics

443 The minimum inhibitory concentration (MIC) of colistin, polymyxin B, cerulenin, ciprofloxacin, EDTA,
444 tetracycline, chloramphenicol (all from Sigma-Aldrich, USA), CHIR090 (Axon Medchem, Netherlands),
445 gentamicin (Thermo Fisher Scientific), and imipenem (Fresenius Kabi, Germany) against bacterial strains was
446 determined by the broth microdilution protocol (Supplementary Table 2, 3) [39]. A microtitre plate was used
447 to prepare a range of antibiotic concentrations in 200 µl Mueller-Hinton broth (MHB) by two-fold serial
448 dilutions. For certain experiments, checkerboard analyses were performed by preparing two-fold serial
449 dilutions of two antibiotics in different directions, generating an 8 x 8 matrix to assess the MICs of the
450 antibiotics in combination [17]. Stationary-phase bacteria were diluted 1000-fold in fresh MHB and seeded
451 into each well of the microtitre plate to a final concentration of 5 x 10^5 c.f.u. ml^{-1}. The microtitre plates were
452 then incubated statically at 37°C for 18 h in air, after which point the MIC was defined as the lowest antibiotic
453 concentration at which there was no visible growth of bacteria. In some cases, the extent of bacterial growth
454 after 18 h incubation was also determined by obtaining OD_{595nm} measurements using a Bio-Rad iMark
455 microplate absorbance reader (Bio-Rad Laboratories, USA).

456 ### Production of spheroplasts

457 Spheroplasts of *P. aeruginosa* and *E. coli* strains lacking an OM and cell wall were generated as previously
458 described [40-42]. Briefly, stationary-phase bacteria grown overnight were washed twice by centrifugation
459 (12,300 x *g*, 3 min) followed by resuspension in MHB, and added at a final inoculum of 10^8 c.f.u. ml^{-1} to 9 ml
460 MHB, containing for some experiments the LPS biosynthesis inhibitors CHIR090 or cerulenin. Cultures were
461 then incubated at 37°C with shaking (180 r.p.m.) for 2 h. After the incubation, bacteria were washed twice

462 by centrifuging (3,273 x *g*, 20 min, 4°C) and resuspending first in 10 ml Tris buffer (0.03 M, pH 8.0, Sigma-
463 Aldrich), and subsequently in Tris buffer (0.03 M, pH 8.0) containing 20% sucrose. EDTA (250 µl, 10 mg ml^{-1})
464 and lysozyme (1 ml, 10 mg ml^{-1}, Roche, Switzerland) were added to remove the OM and cell wall respectively,
465 and the cell suspension was incubated for 1 h in a water bath shaker at 30°C. Trypsin (500 µl, 10 mg ml^{-1},
466 Sigma-Aldrich) was then added, and the culture again incubated at 30°C in a water bath shaker for 15 min.
467 The resulting spheroplasts produced were harvested by mild centrifugation (2,000 x *g*, 20 min, 4°C) and
468 resuspended in 1 ml Tris buffer (0.03 M, pH 8.0) containing 20% sucrose. Successful conversion of bacterial
469 whole cells into spheroplasts was confirmed using phase-contrast microscopy, as detailed below.

470 **Fluorescent labelling of antibiotics**

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

480 **Microscopic analysis**

481 For phase-contrast and fluorescence microscopy, a 5 µl sample of fixed bacterial whole cells or spheroplasts
482 was spotted onto a thin 1.2% agarose gel patch prepared in distilled water on a microscope slide. Bacteria
483 were imaged using an Axio Imager.A2 Zeiss microscope (Carl Zeiss Microscopy GmbH, Germany) at 1000x
484 magnification with an oil immersion objective lens. The ZEN 2012 software was used for image acquisition,
485 whilst analysis of cell length:width ratios was done using the FIJI/ImageJ software by measuring two
486 perpendicular lines drawn through the centre of bacteria. For each experiment, all microscopy images were
487 acquired and processed using identical settings throughout.

488 **Determination of LPS levels on cell surface**

489 To detect and quantify the amount of LPS present in the OM of whole cells and CM of spheroplasts, a
490 fluorescently-labelled form of PMBN (BoDipy-PMBN) was used as a probe, as described previously [25]. For
491 whole cells, stationary-phase bacterial cultures grown overnight were washed twice by centrifugation
492 (12,300 x *g*, 3 min) and resuspension in MHB, before being added at a final inoculum of 10^8 c.f.u. ml^{-1} to 9 ml
493 MHB containing CHIR090 or cerulenin where required. Bacteria were then incubated at 37°C with shaking
494 (180 r.p.m.) for 2 h, after which point cells were washed by centrifuging (3,273 x *g*, 20 min, 4°C) and

495 resuspending in 1 ml Tris buffer (0.03 M, pH 8.0). Washed bacteria were recovered by centrifugation (12,300
496 x *g*, 3 min) and fixed by resuspending in 1 ml of 4% PFA in phosphate-buffered saline (PBS). Similarly,
497 spheroplasts generated as detailed above were harvested by mild centrifugation (4,000 x *g*, 5 min), and fixed
498 in the same way as for whole cells. Following fixation for 1 h at room temperature, both whole cells and
499 spheroplasts were washed twice by centrifuging (12,300 x *g*, 3 min for whole cells; 4,000 x *g*, 5 min for
500 spheroplasts) and resuspending in 1 ml Tris buffer (0.03 M, pH 8.0) containing 20% sucrose in the case of
501 spheroplasts. Subsequently, BoDipy-PMBN (7 µg ml^{-1}) was added, and the fixed bacterial suspensions were
502 incubated with end-over-end rotation at 37°C for 30 min. After the incubation, to remove any BoDipy-PMBN
503 that was not bound to the cell surface, the whole cells and spheroplasts were again washed twice as stated
504 above. The amount of BoDipy-PMBN attached to the OM of whole cells and CM of spheroplasts was observed
505 using fluorescence microscopy, as described above. For quantification of BoDipy-PMBN bound to the cell
506 surface, 200 µl samples of the bacterial suspensions were seeded into the wells of a black-walled microtitre
507 plate, and fluorescence measured with a Tecan Infinite 200 Pro multiwell plate reader (Tecan Group Ltd.,
508 Switzerland), using an excitation wavelength of 490 nm and an emission wavelength of 525 nm. To account
509 for differences in fluorescence values arising from variations in cell number, relative fluorescence unit (r.f.u.)
510 measurements were corrected for OD at 600 nm.

511 **Bacterial growth assay**

512 Stationary-phase bacteria were diluted 1000-fold in fresh MHB, and 4 µl was seeded into the wells of a
513 microtitre plate containing 200 µl MHB, and for some experiments the LPS biosynthesis inhibitors CHIR090
514 or cerulenin, to give a final inoculum of 5 x 10^5 c.f.u. ml^{-1}. The microtitre plate was incubated with shaking
515 (180 r.p.m.) at 37°C for up to 24 h in a Tecan Infinite 200 Pro multiwell plate reader and optical density
516 measurements were taken at 600 nm every 15 min.

517 **Outer membrane disruption assay**

518 To detect damage to the OM of bacteria, the well-established NPN uptake assay was used [27]. Stationary-
519 phase bacterial cells were washed in fresh MHB and diluted to an optical density (OD_{600nm}) of 0.5 in 5 mM pH
520 7.2 HEPES buffer (Sigma-Aldrich). This bacterial suspension was added to wells containing the relevant
521 antibiotics in HEPES buffer, as well as the fluorescent probe *N*-phenyl-1-naphthylamine (NPN; Acros Organics,
522 USA) at a final concentration of 10 µM. Samples were placed in a black microtitre plate with clear-bottomed
523 wells and fluorescence measured immediately in a Tecan Infinite 200 Pro multiwell plate using an excitation
524 wavelength of 355 nm and an emission wavelength of 405 nm. Fluorescence measurements were obtained
525 every 30 seconds for 10 min, and the degree of OM permeabilisation, referred to as the NPN uptake factor,
526 was calculated using the following formula:

527 $$\frac{Fluorescence\ of\ sample\ with\ NPN - Fluorescence\ of\ sample\ without\ NPN}{Fluorescence\ of\ HEPES\ buffer\ with\ NPN - Fluorescence\ of\ HEPES\ buffer\ without\ NPN}$$

528 **Determination of antibiotic binding to bacterial cells**

529 Stationary-phase bacterial cultures grown overnight were washed twice in MHB by centrifugation (12,300 x
530 *g*, 3 min) and resuspension, then added at an inoculum of 10^8 c.f.u. ml^{-1} to 3 ml MHB containing fluorescently-
531 labelled BoDipy-Colistin or BoDipy-Polymyxin B, either alone or in combination with CHIR090 or cerulenin.
532 The cultures were incubated for 8 h at 37°C with shaking (180 r.p.m.), and every 2 h bacteria were recovered
533 by centrifugation (12,300 x *g*, 3 min). The pelleted cells were washed thoroughly by four rounds of
534 centrifugation (12,300 x *g*, 3 min) and resuspension in fresh MHB in order to remove any BoDipy-labelled
535 antibiotic that was not attached to the bacteria. The amount of BoDipy-Colistin or BoDipy-Polymyxin B bound
536 to the bacterial cells was quantified by measuring the fluorescence of 200 µl samples in a black-walled clear-
537 bottomed microtitre plate, using a Tecan Infinite 200 Pro multiwell plate reader (excitation at 490 nm,
538 emission at 525 nm). The relative fluorescence values of samples were corrected using OD_{600nm}
539 measurements.

540 **Determination of bactericidal activity of antibiotics**

541 As described above, stationary-phase bacteria grown overnight were washed twice and added at a final
542 inoculum of 10^8 c.f.u. ml^{-1} to 3 ml MHB containing the relevant antibiotics. Cultures were incubated with
543 shaking (37°C, 180 r.p.m.) for up to 10 h. Bacterial survival was determined after 2, 4, 6, 8 and 10 h by serially
544 diluting cultures in 10-fold steps in 200 µl sterile PBS (VWR International), before enumeration of c.f.u. counts
545 on MHA plates.

546 **Isolation of CHIR090-resistant mutants**

547 Cultures of *P. aeruginosa* grown overnight to stationary-phase were washed twice in MHB as described above
548 and diluted 10-fold to an inoculum of 10^8 c.f.u. ml^{-1} in MHB, before 100 µl of this inoculum was spread across
549 the surface of an MHA plate. A well measuring 10 mm in diameter was made in the agar plate and filled with
550 130 µl of the LPS biosynthesis inhibitor CHIR090 (8 µg ml^{-1}). After static incubation of the agar plate at 37°C
551 for 18 h in air, mutants with spontaneous resistance to CHIR090 were isolated by picking colonies that had
552 grown within the zone of inhibition around the well. These resistant mutants were grown overnight to
553 stationary-phase, and again exposed to CHIR090 in a well as described above to confirm that the strains had
554 a reduced susceptibility to the antibiotic. Bacterial growth, survival and MIC assays were performed as
555 detailed in the relevant sections to characterise the properties of the resistant *P. aeruginosa* isolates.

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

557 Stationary-phase bacteria grown overnight were washed twice in MHB and exposed to the relevant
558 antibiotics in 3 ml MHB at a final inoculum of 10^8 c.f.u. ml^{-1} as described above. Cultures were incubated for
559 8 h at 37°C with shaking (180 r.p.m.), and every 2 h the supernatants were recovered by centrifugation
560 (12,300 x *g*, 3 min). Recovered supernatants (200 µl) were mixed with 5 µl of FM-4-64 styryl dye (Thermo

561 Fisher Scientific) at a final concentration of 5 µg ml^{-1} in the wells of a black-walled microtitre plate.
562 Fluorescence was measured using a Tecan Infinite 200 Pro multiwell plate reader (excitation at 565 nm,
563 emission at 600 nm) to quantify the phospholipid released into the supernatant by bacteria.

564 **Determination of LPS release from bacteria**

565 Culture supernatants from bacteria exposed to the relevant antibiotics were recovered as detailed above,
566 and the chromogenic Limulus Amebocyte Lysate (LAL) assay (all reagents from Thermo Fisher Scientific) was
567 used to quantify LPS released from bacteria into the culture supernatant as described previously [43].
568 Samples of cell-free culture supernatant (50 µl) were equilibrated to 37°C and loaded into the wells of a
569 microtitre plate at the same temperature. Limulus amebocyte lysate reagent (50 µl) was added to each well,
570 and the mixture incubated at 37°C for 10 min. Chromogenic substrate solution (100 µl, 2 mM) was
571 subsequently added to each well and the microtitre plate was incubated for a further 6 min at 37°C. The
572 enzymatic reaction was stopped by adding 50 µl of 25% acetic acid to each well, and the presence of LPS was
573 determined by measuring absorbance at 405 nm in a Tecan Infinite 200 Pro multiwell plate reader. A standard
574 curve was generated using an *E. coli* endotoxin standard stock solution, which enabled the conversion of
575 A_{405nm} values into concentrations of LPS in the supernatant.

576 **Determination of colistin activity**

577 As described above, spent culture supernatants from bacteria exposed to the relevant antibiotics were
578 recovered and the activity of colistin in the supernatant was determined using an established zone of
579 inhibition assay [44]. Stationary-phase *P. aeruginosa* was diluted in MHB to a concentration of 10^6 c.f.u. ml^{-1}
580 before 60 µl of this bacterial culture was spread across the surface of an MHA plate and allowed to air-dry.
581 Wells measuring 10 mm in diameter were made in the agar plate and filled with 130 µl of the culture
582 supernatant recovered from colistin-treated bacterial populations. Agar plates were incubated in air statically
583 at 37°C for 18 h and the zone of growth inhibition around each well was measured at four perpendicular
584 points. A standard plot was generated showing a linear relationship between the size of the zone of inhibition
585 and colistin concentration, which enabled inhibitory zone size to be converted to percentage colistin activity.

586 **Scanning electron microscopy (SEM) imaging**

587 Stationary-phase bacterial cells grown overnight were washed and inoculated into 3 ml MHB containing the
588 relevant antibiotics as described above. After 2 h incubation (37°C, 180 r.p.m.), 25 µl of the cultures was
589 spotted onto a silicon chip (University Wafer, USA) and allowed to air-dry. The sample was fixed using
590 glutaraldehyde (2.5% in 0.01 M PBS) for 30 min at room temperature, before the chip was washed three
591 times in PBS (0.01 M). The sample was dehydrated using increasing concentrations of ethanol (10%, 30%,
592 50%, 70%, 90% and 100%) for 5 min each time, before a coating of 15 nm chromium (Quorum Technologies,

593 UK) was applied. Representative images of bacterial cells were obtained using a LEO Gemini 1525 field
594 emission gun scanning electron microscope (FEG SEM; Carl Zeiss Microscopy GmbH).

595 **Cytoplasmic membrane disruption assay**

596 To measure CM disruption of whole cells, as described above bacteria grown to stationary-phase overnight
597 were washed and inoculated into 3 ml MHB containing the relevant antibiotics. Cultures were incubated at
598 37°C with shaking (180 r.p.m.) for up to 8 h, and every 30 min, aliquots (200 µl) were taken and bacteria
599 isolated by centrifugation (12,300 x *g*, 3 min). Cells were then washed in sterile PBS before being added to
600 the wells of a black-walled microtitre plate, and propidium iodide (PI, Sigma-Aldrich) was added to each well
601 at a final concentration of 2.5 µM. Fluorescence was measured immediately in a Tecan Infinite 200 Pro
602 multiwell plate reader (excitation at 535 nm, emission at 617 nm). To measure disruption of the CM in
603 spheroplasts, spheroplasts of *P. aeruginosa* and *E. coli* generated as detailed above were washed by
604 centrifugation (4,000 x *g*, 5 min) and resuspension in Tris buffer (0.03 M, pH 8.0) containing 20% sucrose.
605 Spheroplast samples (20 µl) were then added in the wells of a black-walled microtitre plate to 180 µl of Tris
606 buffer (0.03 M, pH 8.0) containing 20% sucrose, the relevant antibiotics, and PI at a final concentration of
607 0.25 µM. The microtitre plate was incubated with shaking (180 r.p.m.) at 37°C for up to 12 h in a Tecan Infinite
608 200 Pro multiwell plate reader and fluorescence (excitation at 535 nm, emission at 617 nm) measured every
609 15 min. For both whole bacterial cells and spheroplasts, to account for differences in fluorescence values
610 arising from variations in cell number, relative fluorescence unit (r.f.u.) measurements were corrected for
611 OD at 600 nm.

612 **Determination of bacterial lysis**

613 In the case of whole bacterial cells, washed stationary-phase bacteria were inoculated into 3 ml MHB
614 containing the relevant antibiotics, as described above. Cultures were then placed in a shaking incubator
615 (37°C, 180 r.p.m.) for 8 h, and every 30 min, samples (200 µl) were transferred to a microtitre plate, where
616 OD_{595nm} measurements were obtained using a Bio-Rad iMark microplate absorbance reader. For
617 spheroplasts, washed spheroplasts (20 µl) were added to 180 µl of Tris buffer (0.03 M, pH 8.0) containing
618 20% sucrose and the relevant antibiotics in a microtitre plate as detailed above. The microtitre plate was
619 incubated for up to 12 h at 37°C with shaking (180 r.p.m.) in a Tecan Infinite 200 Pro multiwell plate reader,
620 and readings of OD_{600nm} were made every 15 min.

621 **Statistical analyses**

622 Experiments were performed on at least three independent occasions, and the resulting data are presented
623 as the arithmetic mean of these biological repeats, unless stated otherwise. Error bars represent the standard
624 deviation of the mean. For single comparisons, a two-tailed unpaired Student's *t*-test was used to analyse
625 the data. For multiple comparisons at a single time point or concentration, data were analysed using a one-

way analysis of variance (ANOVA). Where data were obtained at several different time points or concentrations, a two-way ANOVA was used for statistical analyses. Appropriate post-hoc tests (Dunnett's, Tukey's, Welch's, Sidak's, Holm-Sidak's) were carried out to correct for multiple comparisons, with details provided in the figure legends. Asterisks on graphs indicate significant differences between data, and the corresponding p-values are reported in the figure legend. All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., USA).

Data availability

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

Acknowledgements

Aishwarya Krishna, Laura Nolan, Alain Filloux (all Imperial College London) and Alireza Abdolrasouli (Imperial College Healthcare NHS Trust) are gratefully acknowledged for providing bacterial strains. Lisa Haigh (Imperial College London) is thanked for processing and analysing mass spectrometry samples. Vladimir Pelicic (Imperial College London) is thanked for providing access to the Axio Imager.A2 Zeiss microscope. 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). D.A.I.M. and R.C.F. are supported by an MRC Career Development Award (MR/M009505/1). 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., M.B., R.C.D.F. performed experiments or generated bacterial strains. All authors analysed data and contributed to writing of the manuscript.

Competing interests

The authors declare no competing interests.

682 **References**

683 1. Velkov, T., Thompson, P. E., Nation, R. L., Li, J. Structure--activity relationships of polymyxin antibiotics. *J.*
684 *Med. Chem.* **53(5),** 1898-1916 (2010).
685 2. Garg, S. K. *et al*. Resurgence of Polymyxin B for MDR/XDR Gram-Negative Infections: An Overview of
686 Current Evidence. *Crit. Care. Res. Pract*. 3635609 (2017).
687 3. Biswas, S., Brunel, J. M., Dubus, J. C., Reynaud-Gaubert, M., Rolain, J. M. Colistin: an update on the antibiotic
688 of the 21st century. *Expert Rev. Anti. Infect. Ther.* **10(8),** 917-934 (2012).
689 4. Thomas, R. *et al*. The use of polymyxins to treat carbapenem resistant infections in neonates and children.
690 *Expert Opin. Pharmacother*. **20(4),** 415-422. (2019).
691 5. Falagas, M. E. *et al.* Colistin therapy for microbiologically documented multidrug-resistant Gram-negative
692 bacterial infections: a retrospective cohort study of 258 patients. *Int. J. Antimicrob. Agents.* **35(2),** 194-199
693 (2010).
694 6. Paul, M. *et al*. Colistin alone versus colistin plus meropenem for treatment of severe infections caused by
695 carbapenem-resistant Gram-negative bacteria: an open-label, randomised controlled trial. *Lancet Infect.*
696 *Dis*. **18(4),** 391-400 (2018).
697 7. Linden, P. K. *et al.* Use of parenteral colistin for the treatment of serious infection due to antimicrobial-
698 resistant *Pseudomonas aeruginosa*. *Clin. Infect. Dis.* **37(11),** e154-160 (2003).
699 8. Gales, A. C., Jones, R. N., Sader, H. S. Contemporary activity of colistin and polymyxin B against a worldwide
700 collection of Gram-negative pathogens: results from the SENTRY Antimicrobial Surveillance Program (2006-
701 09). *J. Antimicrob. Chemother.* **66(9),** 2070-2074 (2011).
702 9. Poirel, L., Jayol, A., Nordmann, P. Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance
703 Mechanisms Encoded by Plasmids or Chromosomes. *Clin. Microbiol. Rev*. **30(2),** 557-596 (2017).
704 10. Simpson, B. W., Trent, M. S. Pushing the envelope: LPS modifications and their consequences. *Nat. Rev.*
705 *Microbiol.* **17(7),** 403-416 (2019).
706 11. Liu, Y. Y. *et al*. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human
707 beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis*. **16(2),** 161-168 (2016).
708 12. Carroll, L. M. *et al.* Identification of Novel Mobilized Colistin Resistance Gene *mcr-9* in a Multidrug-
709 Resistant, Colistin-Susceptible *Salmonella enterica* Serotype Typhimurium Isolate. *MBio.* **10(3),** e00853-19
710 (2019).
711 13. Nang, S. C., Li, J., Velkov, T. The rise and spread of *mcr* plasmid-mediated polymyxin resistance. *Crit. Rev.*
712 *Microbiol.* **45 (2),** 131-161 (2019).
713 14. Skov, R. L., Monnet, D. L. Plasmid-mediated colistin resistance (*mcr*-1 gene): three months later, the story
714 unfolds. *Euro. Surveill.* **21(9),** 30155 (2016).
715 15. Moffatt, J. H. *et al*. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of
716 lipopolysaccharide production. *Antimicrob. Agents Chemother*. **54(12),** 4971-4977 (2010).

717 16. McClerren, A. L. *et al*. A slow, tight-binding inhibitor of the zinc-dependent deacetylase LpxC of lipid A
718 biosynthesis with antibiotic activity comparable to ciprofloxacin. *Biochemistry*. **44(50),** 16574-16583
719 (2005).
720 17. MacNair, C. R. *et al.* Overcoming *mcr-1* mediated colistin resistance with colistin in combination with other
721 antibiotics. *Nat. Commun.* **9(1),** 458 (2018).
722 18. Powers, J. P. S., Hancock, R. E. W. The relationship between peptide structure and antibacterial activity.
723 *Peptides* **24(11),** 1681–1691 (2003).
724 19. Halebian, S., Harris, B., Finegold, S. M., Rolfe, R. D. Rapid method that aids in distinguishing Gram-positive
725 from Gram-negative anaerobic bacteria. *J. Clin. Microbiol*. **13(3),** 444-448 (1981).
726 20. Khadka, N. K., Aryal, C. M., Pan, J. Lipopolysaccharide-Dependent Membrane Permeation and Lipid
727 Clustering Caused by Cyclic Lipopeptide Colistin. *ACS Omega*. **3(12),** 17828-17834 (2018).
728 21. Okuda, S., Sherman, D. J., Silhavy, T. J., Ruiz, N., Kahne, D. Lipopolysaccharide transport and assembly at
729 the outer membrane: the PEZ model. *Nat. Rev. Microbiol*. **14(6),** 337-345 (2016).
730 22. Zhou, Z., White, K. A., Polissi, A., Georgopoulos, C., Raetz, C. R. Function of *Escherichia coli* MsbA, an
731 essential ABC family transporter, in lipid A and phospholipid biosynthesis. *J. Biol. Chem*. **273(20),** 12466-
732 12475 (1998).
733 23. Owens, T. W. *et al*. Structural basis of unidirectional export of lipopolysaccharide to the cell surface. *Nature*.
734 **567(7749),** 550-553 (2019).
735 24. Li, Y., Orlando, B. J., Liao, M. Structural basis of lipopolysaccharide extraction by the $LptB_2FGC$ complex.
736 *Nature*. **567(7749),** 486-490 (2019).
737 25. Moison, E. *et al.* A Fluorescent Probe Distinguishes between Inhibition of Early and Late Steps of
738 Lipopolysaccharide Biogenesis in Whole Cells. *ACS Chem. Biol.* **12(4),** 928-932 (2017).
739 26. Goldberg, I., Walker, J. R., Bloch, K. Inhibition of lipid synthesis in *Escherichia coli* cells by the antibiotic
740 cerulenin. *Antimicrob. Agents Chemother*. **3(5),** 549-554 (1973).
741 27. Helander, I. M., Mattila-Sandholm, T. Fluorometric assessment of gram-negative bacterial
742 permeabilization. *J. Appl. Microbiol.* **88(2),** 213-219 (2000).
743 28. Macfarlane, E. L., Kwasnicka, A., Ochs, M. M., Hancock, R. E. PhoP-PhoQ homologues in *Pseudomonas*
744 *aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Mol.*
745 *Microbiol.* **34(2),** 305-316 (1999).
746 29. Schurek, K. N. *et al.* Involvement of *pmrAB* and *phoPQ* in polymyxin B adaptation and inducible resistance
747 in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother*. **53(10),**
748 4345-4351 (2009).
749 30. Miller, A. K. *et al*. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas*
750 *aeruginosa* found in colistin-treated cystic fibrosis patients. *Antimicrob. Agents Chemother*. **55(12),** 5761-
751 5769 (2011).

752 31. Evans, D. J., Brown, M. R., Allison, D. G., Gilbert, P. Susceptibility of bacterial biofilms to tobramycin: role of
753 specific growth rate and phase in the division cycle. *J. Antimicrob. Chemother.* **25(4),** 585-591 (1990).
754 32. Lee, A. J. *et al.* Robust, linear correlations between growth rates and β-lactam-mediated lysis rates. *Proc.*
755 *Natl. Acad. Sci. U. S. A.* **115(16),** 4069-4074 (2018).
756 33. Manning, A. J., Kuehn, M. J. Contribution of bacterial outer membrane vesicles to innate bacterial defense.
757 *BMC Microbiol.* **11,** 258 (2011).
758 34. Pietschmann, S., Hoffmann, K., Voget, M., Pison, U. Synergistic effects of miconazole and polymyxin B on
759 microbial pathogens. *Vet. Res. Commun*. **33(6),** 489-505 (2009).
760 35. Doerrler, W. T., Gibbons, H. S., Raetz, C. R. MsbA-dependent translocation of lipids across the inner
761 membrane of *Escherichia coli*. *J. Biol. Chem.* **279(43),** 45102-45109 (2004).
762 36. Dortet, L. *et al.* Rapid detection and discrimination of chromosome- and MCR-plasmid-mediated resistance
763 to polymyxins by MALDI-TOF MS in *Escherichia coli*: the MALDIxin test. *J. Antimicrob. Chemother.* **73(12),**
764 3359-3367 (2018).
765 37. Deris, Z. Z. *et al*. Probing the penetration of antimicrobial polymyxin lipopeptides into gram-negative
766 bacteria. *Bioconjug. Chem*. **25(4),** 750-760 (2014).
767 38. Han, M. L. *et al*., Polymyxin-Induced Lipid A Deacylation in *Pseudomonas aeruginosa* Perturbs Polymyxin
768 Penetration and Confers High-Level Resistance. *ACS Chem. Biol*. **13(1),** 121-130 (2018).
769 39. Wiegand, I., Hilpert, K., Hancock, R. E. Agar and broth dilution methods to determine the minimal inhibitory
770 concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **3(2),** 163-175 (2008).
771 40. Weiss, R. L., Fraser, D. Surface Structure of Intact Cells and Spheroplasts of *Pseudomonas aeruginosa*. *J.*
772 *Bacteriol.* **113(2),** 963-968 (1973).
773 41. Cheng, K. J., Costerton, J. W., Singh, A. P., Ingram, J. M., Idziak, E. S. Susceptibility of Whole Cells and
774 Spheroplasts of *Pseudomonas aeruginosa* to Actinomycin D. *Antimicrob. Agents Chemother.* **3(3),** 399-406
775 (1973).
776 42. Guérin-Méchin, L., Leveau J. Y., Dubois-Brissonnet F. Resistance of spheroplasts and whole cells of
777 *Pseudomonas aeruginosa* to bactericidal activity of various biocides: evidence of the membrane
778 implication. *Microbiol. Res.* **159(1)**, 51-57 (2004).
779 43. Roslansky, P. F., Novitsky, T. J. Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans. *J. Clin.*
780 *Microbiol.* **29(11),** 2477-2483 (1991).
781 44. Pader, V. *et al. Staphylococcus aureus* inactivates daptomycin by releasing membrane phospholipids. *Nat.*
782 *Microbiol.* **2,** 16194 (2016).