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1 **The antimicrobial activity of a carbon monoxide releasing molecule**  
2 **(EBOR-CORM-1) is shaped by intraspecific variation within**  
3 ***Pseudomonas aeruginosa* populations**

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13 **ABSTRACT**

14 Carbon monoxide releasing molecules (CORMs) have been suggested as a new synthetic  
15 class of antimicrobials to treat bacterial infections. Here we utilised a novel EBOR-CORM-1  
16 ( $[\text{NEt}_4][\text{MnBr}_2(\text{CO})_4]$ ) capable of water-triggered CO-release, and tested its efficacy against a  
17 collection of clinical *Pseudomonas aeruginosa* strains that differ in infection-related  
18 virulence traits. We found that while EBOR-CORM-1 was effective in clearing planktonic  
19 and biofilm cells of *P. aeruginosa* strain PAO1 in a concentration dependent manner, this  
20 effect was less clear and varied considerably between different *P. aeruginosa* cystic fibrosis  
21 (CF) lung isolates. While a reduction in cell growth was observed after 8 hours of CORM  
22 application, either no effect or even a slight increase in cell densities and the amount of  
23 biofilm was observed after 24 hours. This variation could be partly explained by differences  
24 in bacterial virulence traits: while CF isolates showed attenuated *in vivo* virulence and growth  
25 compared to strain PAO1, they formed much more biofilm, which could have potentially  
26 protected them from the CORM. Even though no clear therapeutic benefits against a subset of  
27 isolates was observed in an *in vivo* wax moth acute infection model, EBOR-CORM-1 was  
28 more efficient at reducing the growth of CF isolate co-culture populations harbouring  
29 intraspecific variation, in comparison with efficacy against more uniform single isolate  
30 culture populations. Together these results suggest that CORMs could be effective at  
31 controlling genetically diverse *P. aeruginosa* populations typical for natural chronic CF  
32 infections and that the potential benefits of some antibiotics might not be observed if tested  
33 only against clonal bacterial populations.

34

35 **Keywords:** Biofilms, Carbon monoxide releasing molecules, CORM, Cystic fibrosis,  
36 Polymicrobial infections, *Pseudomonas aeruginosa*, Synthetic chemistry, Virulence

## 37 1. INTRODUCTION

38 The rapid emergence of multidrug-resistant bacteria is a global problem that is predicted to  
39 cause ten million deaths per year by 2050 (O'Neill, 2014). Antibiotic resistance often evolves  
40 very quickly via *de novo* mutations and horizontal gene transfer (Normark and Normark,  
41 2002), and as a result, antibiotic discovery has not been able to replace all of the antibiotics  
42 that have now become ineffective (Brown and Wright, 2016). New methods and approaches  
43 for treating bacterial infections are thus urgently required.

44 In recent years, carbon monoxide has emerged as a new potential therapeutic due to its  
45 properties as a homeostatic and cytoprotective molecule with important signalling  
46 capabilities (Motterlini and Otterbein, 2010). Carbon monoxide can be delivered via carbon  
47 monoxide releasing molecules (CORMs), which are small molecules that release carbon  
48 monoxide in response to certain environmental triggers such as enzymes (Stamellou et al.,  
49 2014) or light (Jimenez et al., 2016). Nobre *et al.* first investigated the effect of CORMs on  
50 bacteria (Nobre et al., 2007) and found that CORM-2 and CORM-3 reduced the number of  
51 colony-forming units of *Escherichia coli* in minimal salts media and *Staphylococcus aureus*  
52 in Luria Broth (LB) media (Nobre et al., 2007). The CORM effects were stronger in near-  
53 anaerobic conditions and the activation of CORM required direct contact between the  
54 molecule and its cellular targets (Nobre et al., 2007). Moreover, the effect of CORM-3 on  
55 *Pseudomonas aeruginosa* wild type strain PAO1 was investigated by Desmard *et al.*  
56 (Desmard et al., 2009), who found that treatment with the CORM reduced bacterial densities  
57 and increased the survival of immunocompromised mice during an infection. It has also  
58 previously been found that CORM-2 effectively reduces the densities of *P. aeruginosa*  
59 planktonic and biofilm cultures with wild type and clinical strains (Murray et al., 2012).  
60 Another study found that manganese-based Trypto-CORM is able to inhibit the growth of *E.*  
61 *coli* when exposed to photochemical stimulus (Ward et al., 2014), while in the dark it is  
62 active against *Neisseria gonorrhoeae* (Ward et al., 2017). In both cases, control experiments  
63 indicate that the CO liberated from the metal is responsible for the observed behaviour.  
64 However, most of the studies thus far have concentrated on exploring CORM effects on  
65 relatively short time span (less than 24 hours). Furthermore, although it has been established  
66 that many infections are polymicrobial, and that clinical bacterial pathogens can respond  
67 differently to CORMs than laboratory strains, no studies have explored CORM effects on  
68 bacterial co-cultures.

69 Cystic fibrosis (CF) is a genetically inherited disease which affects 1 in 2000 to 3000  
70 newborn infants in the EU (who.int, 2010). Patients with CF often develop a thick mucus in  
71 the lungs which they are unable to clear (Flume et al., 2010). This mucus makes patients  
72 susceptible to frequent and recurring bacterial chest infections and the presence of *P.*  
73 *aeruginosa* is often associated with increasing morbidity and loss of lung function (Pritt et  
74 al., 2007). One of the key features of *P. aeruginosa* is its capability to rapidly adapt to the  
75 lung environment and to become highly resistant to the antibiotics that are used to treat  
76 infections (Smith et al., 2006;Poole, 2011;Folkesson et al., 2012;Winstanley et al., 2016). As  
77 a result, *P. aeruginosa* populations show high levels of genetic variation within and between  
78 CF patients (Marvig et al., 2013;Williams et al., 2015;O'Brien et al., 2017). This includes  
79 phenotypic and genomic heterogeneity within genetically-related populations of *P.*  
80 *aeruginosa* derived from the same clonal lineage (Mowat et al., 2011;Workentine et al.,  
81 2013;Williams et al., 2015). This variation might also affect the applicability of potential  
82 alternative therapies if it is linked with bacterial life-history traits that relate to potential  
83 resistance mechanisms.

84 Here we synthesised and characterised a water-soluble CORM (EBOR-CORM-1),  
85  $[\text{NEt}_4][\text{MnBr}_2(\text{CO})_4]$ , and tested its effectiveness against *P. aeruginosa* strain PAO1 and a  
86 selection of *P. aeruginosa* CF isolates originating from a single sputum sample from the  
87 lungs of a CF patient, namely patient CF03 from previously published studies (Mowat et al.,  
88 2011;Williams et al., 2015). Based on genome sequence data presented in a previous study,  
89 these CF isolates were classified into two genetically distinct Liverpool Epidemic Strain  
90 (LES) lineages, A and B (Williams et al., 2015;Williams et al., 2016), that differ regarding  
91 their virulence traits (O'Brien et al., 2017). These genetically diverged lineages have been  
92 shown to commonly coexist within individual patients and to share mutations via  
93 homologous recombination that potentially help strains to adapt to the airway during chronic  
94 infection (Williams et al., 2015). However, the implications of within-patient genetic  
95 variation have been seldom considered in the context of antimicrobial therapies. We  
96 hypothesised that effects of EBOR-CORM-1 could vary between different clinical isolates  
97 and lineages, and that the susceptibility of isolates could be linked to expression of some  
98 other bacterial virulence factors. We found that the CORM was effective in reducing both  
99 planktonic and biofilm cells of strain PAO1 in a density-dependent manner. However,  
100 CORM effects were more varied and generally weaker against clinical CF isolates.  
101 Regardless, CORM efficiently reduced the growth of CF strain lineage co-cultures, which  
102 suggest that CORMs could be effective at controlling genetically diverse *P. aeruginosa*  
103 infections.

## 104 2. MATERIALS AND METHODS

### 105 ***Synthesis and properties of [NEt<sub>4</sub>][MnBr<sub>2</sub>(CO)<sub>4</sub>], EBOR-CORM-1***

106 EBOR-CORM-1 was synthesised as described previously (Angelici, 1964): Mn(CO)<sub>5</sub>Br (466  
107 mg, 1.69 mmol) and 330 mg (1.57 mmol) of [(C<sub>2</sub>H<sub>6</sub>)<sub>4</sub>N]Br were heated in 18 mL of absolute  
108 methanol under a nitrogen atmosphere at 50 °C for 1 hour. The methanol was then  
109 evaporated from the orange solution at the above temperature. The remaining yellow solid  
110 was dissolved in 40 mL of chloroform, and the solution was filtered under nitrogen. After  
111 adding 200 mL of hexane to the filtrate, the cloudy solution was allowed to stand under  
112 nitrogen for 2 hours. The air-stable yellow crystals were separated by filtration, washed with  
113 hexane, and dried under vacuum giving a yield of 88 % (636 mg). The compound was  
114 characterised *via* solid state IR spectroscopy recorded using a KBr disk. Four main bands  
115 were seen at 2090, 2001, 1984 and 1942 cm<sup>-1</sup> and a small shoulder was seen at 1897 cm<sup>-1</sup>.  
116 This is consistent with the literature values (Angelici, 1964). In a chloroform solution of  
117 CORM four distinct bands were observed at 2092, 2015, 1987 and 1943 cm<sup>-1</sup>, again this is  
118 similar to previously reported literature values (Angelici, 1964). The change in the number of  
119 carbonyl bands between the solid and solution phase measurements typically reflects that  
120 different orientations are present in the solid state. The stability of the CORM in the solid  
121 state was tested by heating a sample to 50 °C and running ATR IR spectra at 1 hour intervals.

122 Infrared detection of CO release from EBOR-CORM-1 following dissolution in different  
123 solvents was conducted by dissolving 12 mg of CORM in 4 mL of solvent in a 25 mL round  
124 bottomed flask attached to vacuum evacuated gas IR cell via a closed tap. After 1 h of stirring  
125 the flask, the tap was opened to enable gas from the headspace of the flask to enter the IR  
126 cell. Carbon monoxide could then be identified via the distinctive gaseous IR signature of a  
127 double band, with fine rotational splitting, centred at 2150 cm<sup>-1</sup> (Klein et al., 2014). The  
128 impact of different solvents can be quantified by comparison of the intensity of the CO bands  
129 to those from CO<sub>2</sub>, which is assumed to act as an effective internal standard.

130 The release of CO from EBOR-CORM-1 following dissolution in water was also followed via  
131 solution phase monitoring of the metal complex's IR bands. In contrast to chloroform, when  
132 EBOR-CORM-1 was first dissolved in water only two main IR bands were observed at 2050  
133 and 1943 cm<sup>-1</sup>. In order to investigate activity of EBOR-CORM-1 in liquid culture media, we  
134 compared the effects of active and 'inactivated' CORM on the growth of PAO1 strain in LB  
135 media as described previously (Murray et al., 2012). Briefly, CORM was inactivated by  
136 storing a 2 mM CORM stock LB solution (10% v/v of standard LB concentration, i.e., the  
137 same that was used in all the experiments; see below) at room temperature for 24 hours. To

138 estimate the effect of CORM inactivation on PAO1 growth, we added 50  $\mu$ L of freshly  
139 prepared 2 mM CORM, 50  $\mu$ L of inactivated 2 mM CORM or 50  $\mu$ L 10% v/v LB (control) to  
140 150  $\mu$ L of PAO1 starter culture on 96-well microplate. All treatments were replicated five  
141 times and PAO1 growth monitored for 8 hours at 37 °C with spectrophotometer (OD 600 nm;  
142 Tecan Infinite).

### 143 ***Bacterial strains and culture media***

144 In this study we used *P. aeruginosa* strain PAO1 (ATCC 15692), the earliest archived isolate  
145 of the Liverpool Epidemic strain, LESB58 (Winstanley et al., 2009), and 19 clinical *P.*  
146 *aeruginosa* LES isolates from the same sputum sample of a chronically infected CF patient  
147 (Williams et al., 2015). The CF lung LES isolates originate from the sputum sample of one  
148 patient, identified as patient CF03 in previous studies, and consist of two genetically separate  
149 lineages A and B (Williams et al., 2015). Lineage A was represented by six isolates, namely  
150 isolates: 2, 5, 10, 19, 23 and 25. Lineage B was represented by 13 isolates, namely isolates: 1,  
151 6, 8, 17, 24, 26, 28, 32, 33, 34, 35, 36 and 37. Clinical isolates were collected with the  
152 consent of the patient and under institutional human investigation approval. All strains and  
153 isolates of *P. aeruginosa* were routinely cultured in liquid or solid LB media containing 10.0  
154 g tryptone, 5.0 g yeast extract and 10.0 g NaCl in 1 L of ultra-pure water (final pH adjusted to  
155 7.0 and 15 g of agar was used for solid media). For all experiments, starter cultures were  
156 prepared from cryofrozen stocks by streaking frozen stock culture onto LB plates. After 24  
157 hours growth, a single colony was selected and inoculated into 5 mL of liquid LB and grown  
158 overnight in a shaking incubator at 37 °C in 50 mL centrifuge tubes. Overnight cultures were  
159 centrifuged at 4000 rpm (11.5 g) for 15 min (Eppendorf), the resultant pellets were suspended  
160 in 10% LB and bacterial densities adjusted to optical density at 600 nm of 0.066 before use  
161 (OD 600nm), equalling  $\sim 1 \times 10^8$  cells mL<sup>-1</sup>.

### 162 ***Measuring the effects of EBOR-CORM-1 concentration on P. aeruginosa PAO1*** 163 ***strain***

164 We measured the effect of CORM concentration on *P. aeruginosa* PAO1 in four different  
165 ways. First, we examined how EBOR-CORM-1 affects PAO1 growth after both 8 and 24  
166 hours of inoculation in 10% LB media (bacteria and CORM inoculated at the same time).  
167 Additionally, we measured how effective EBOR-CORM-1 is at clearing both established  
168 planktonic and biofilm PAO1 cultures (bacteria pre-grown before adding EBOR-CORM-1).  
169 All measurements were conducted on 96-well microplates and each treatment was replicated  
170 5 times. A variety of EBOR-CORM-1 concentrations were tested by first preparing a 4 mM  
171 CORM stock solution (dissolving EBOR-CORM-1 in 10% LB media by vortexing for 30 s

172 and sonicating for 1.5 min). The stock solution was then sterilised with syringe filtration and  
173 serially diluted to result in 1 mM, 0.5 mM, 0.25 mM, 0.125 mM and 0 mM (control) EBOR-  
174 CORM-1 concentrations and  $1 \times 10^8$  PAO1 cells mL<sup>-1</sup> with final volume of 200 µl of media.  
175 The microplate was then incubated at 37 °C for 24 hours.

176 All replicate populations were sampled at 8 and 24 hours after the start of the experiment (20  
177 µl of samples) and serially diluted in sterile PBS on microplates to quantify the number of  
178 living versus dead cells by flow cytometry. Briefly, DAPI (4',6-diamidino-2-phenylindole for  
179 dead and living cells) and PI (Propidium iodide for dead cells) fluorescent stains (both from  
180 Sigma-Aldrich) were added to microplate wells with diluted bacterial samples at  
181 concentrations of 1 µg/mL and 50 µM, respectively. Plates were then incubated at room  
182 temperature for 1 hour before measuring cell densities with a Cytoflex flow cytometer and  
183 the CytExpert program. Every well was sampled for 60 s at fast speed setting. Gating of live  
184 and dead cells was performed by monitoring DAPI staining on the PB450 channel with the  
185 405 nm laser, and PI staining on the ECD channel of the 488 nm laser. Number of living cells  
186 was determined as total cells (DAPI) – dead cells (PI).

187 To quantify the effects of EBOR-CORM-1 on established planktonic and biofilm cultures,  
188 PAO1 was first grown in the absence of CORM at 37 °C for 48 hours. Cell cultures were then  
189 inoculated with stock CORM solution to reach the same final concentrations as above: 1  
190 mM, 0.5 mM, 0.25 mM, 0.125 mM and 0 mM (control) of CORM. The plate was incubated  
191 for four more hours at 37 °C before sampling (20 µL), serial dilution and flow cytometry as  
192 described above. To quantify effects of EBOR-CORM-1 on biofilm, crystal violet was added  
193 to the remaining cell cultures at 10% v/v. After 15 min of incubation, the plate was rinsed  
194 with deionised water and solubilised with 228 µL ethanol per well. The biofilm was  
195 quantified by measuring absorbance at 600 nm.

### 196 ***Measuring the effects of EBOR-CORM-1 on clinical P. aeruginosa isolates in*** 197 ***mono- and co-cultures***

198 Similar to the PAO1 strain experiments, we measured the effect of EBOR-CORM-1 on  
199 clinical *P. aeruginosa* isolates after 8 and 24 hours of inoculation in 10% LB media. We also  
200 measured the impact of growing the isolates in the absence of EBOR-CORM-1 for 48 hours  
201 and then applying EBOR-CORM-1 for 4 hours using both flow cytometry and crystal violet  
202 staining. We used only one EBOR-CORM-1 concentration, 0.5 mM, which resulted in clear  
203 reduction of PAO1 cultures (see results) alongside control treatment (no CORM).

204 In addition to measuring the effects of EBOR-CORM-1 in monocultures of each clinical  
205 isolate, we also quantified the effect of the CORM on mixtures of the CF clinical isolates  
206 from patient CF03. First, we prepared the clinical isolate starter cultures as described above,  
207 then we mixed the standardised monocultures together in three different ways: as a whole  
208 mix (all isolates mixed together in equal proportions), lineage A mix (all isolates classified as  
209 lineage A mixed together in equal proportions) and lineage B mix (all isolates classified as  
210 lineage B mixed together in equal proportions). All final mixes contained approximately  $1 \times$   
211  $10^8$  cells  $\text{mL}^{-1}$  before the application of 0.5 mM of EBOR-CORM-1. Each experiment was  
212 replicated 5 times. After 24 hours growth at 37 °C, bacterial densities were measured by  
213 using a Tecan infinite spectrophotometer: optical density measurements correlate well with  
214 the proportion of living cells measured with flow cytometer (Supplementary figure 1).

### 215 ***Characterising bacterial virulence and growth***

216 To characterise production of the virulence factors pyocyanin and pyoverdine, all clinical  
217 isolates were grown in 200  $\mu\text{L}$  of 10% LB media in round-bottomed 96-well microplates for  
218 48 hours at 37 °C (no shaking). After incubation, we measured the bacterial densities (OD  
219 600 nm) and centrifuged the microplate for 10 min. at 4000 rpm (11.5 g) in a swing rotor  
220 Eppendorf centrifuge. To measure pyocyanin and pyoverdine production, 150  $\mu\text{L}$  of the  
221 supernatant of each well was transferred to flat-bottomed 96-well microplates and the  
222 absorbance spectrum measured with a spectrophotometer (Tecan infinite). Per capita  
223 pyocyanin production was measured for each isolate by measuring the absorbance of  
224 supernatant at 691 nm, and then standardizing by bacterial OD (Reszka et al., 2004). Per  
225 capita production of the iron-chelating siderophore, pyoverdine, was measured by using  
226 excitation-emission assay (O'Brien et al., 2017) where the fluorescence of each supernatant  
227 well was measured at 470 nm following excitation at 380 nm, using a Tecan infinite M200  
228 pro spectrophotometer. Also, OD was measured at 600 nm to quantify the ratio  
229 fluorescence/OD as a quantitative measure of per capita pyoverdine production (O'Brien et  
230 al., 2017). The isolate biofilm production was measured as described previously and growth  
231 as maximum density and growth rate  $\text{h}^{-1}$  during 24-hour growth period. Lastly, we also  
232 measured the *in vivo* virulence of each isolate by using wax moth model as described  
233 previously (O'Brien et al., 2017).

234

### 235 ***Testing EBOR-CORM-1 antimicrobial activity in wax moth model in vivo***

236 To test the efficacy of EBOR-CORM-1 to constrain bacterial infections *in vivo*, we used a  
237 wax moth larvae model (*Galleria mellonella* [Lepidoptera: Pyralidae], Livefood UK Ltd) and  
238 followed the infection methodology described previously (O'Brien et al., 2017). We chose  
239 three strains for infection experiments: PAO1, LESB58 and isolate 36 (Lineage B) from the  
240 clinical sample collection. Before infection, we first grew the selected *P. aeruginosa* isolates  
241 for 24 hours at 37 °C and subsequently diluted all cultures to approximately similar densities  
242 (equalling approximately  $1 \times 10^6$  cells mL<sup>-1</sup> in 0.8% w:v NaCl). The virulence of every  
243 isolate was then tested in 16 independent wax moth larvae. We also infected 16 larvae with  
244 0.8% w/v NaCl salt solution to control for the damage caused by the injection itself. The  
245 larvae were injected with either 20 µL of one bacterial solution or NaCl buffer (“non-  
246 infected”) between the abdominal segments six and seven with a 1 mL Terumo syringe. After  
247 2 hours, 8 larvae from each bacterial infection or non-infection group were treated with 20  
248 µL injection of 500 µM EBOR-CORM-1, and the other 8 were injected with 0.8% w:v NaCl  
249 salt solution (control placebo) in the same location where the bacteria were originally  
250 injected. After infection, larvae were placed on individual wells of 24-well cell culture plates  
251 and the survival was monitored at 2-hour intervals for 3 days at 37 °C. Larvae were scored as  
252 dead when they did not respond to touch with forceps. Larvae that were still alive after 7 days  
253 from the infection were given a time of death of 168 hours. Every bacterial isolate was tested  
254 for three times. It was concluded that the EBOR-CORM-1 injection alone did not affect  
255 larval survival in the absence of bacteria (mortality similar between non-infected CORM-  
256 injected larvae and non-infected CORM-free larvae: 5-10%).

### 257 **Statistical analysis**

258 All data were analysed with Generalized Mixed Models (factorial ANOVA) or regression  
259 analysis where bacterial densities (Figs. 2, 3 and 4b) or trait values (Fig. 4a; Supplementary  
260 figure 4) were explained with the presence and/or concentration of EBOR-CORM-1, CF  
261 isolate identity (isolate number) or CF lineage (A or B). All proportional data (%) were  
262 arcsine transformed before the analysis to meet the assumptions of parametric models.

## 263 **3. RESULTS**

### 264 **Chemistry of EBOR-CORM-1**

265 The stability of EBOR-CORM-1 in the solid state was demonstrated by heating a sample of  
266 solid to 50 °C in air, and showing that there is very little difference in the carbonyl bands  
267 observed in ATR IR spectra measured at 1 hour intervals over a 3-hour period (Fig. 1A). In  
268 contrast, gas phase infrared analysis proved that CO release from EBOR-CORM-1 can be

269 triggered by dissolution in water, phosphate buffer or LB media, or addition of water to a  
270 solution of the compound in an organic solvent (Fig. 1B).

271 Solution phase monitoring of the CO stretches of the compound showed that there was no  
272 reaction with water over short periods of time, since dissolving EBOR-CORM-1 in water,  
273 immediately re-drying it on a vacuum line and then re-dissolving the resultant solid in  
274 chloroform yielded an IR spectra which matched that of the as-purified compound in  
275 chloroform (Fig. 1C). The only two observed IR bands in the CORM spectrum in water  
276 (2050 and 1943  $\text{cm}^{-1}$ ) were therefore attributed to the molecular symmetry of the hydrated  
277 complex, rather than an immediate loss of CO upon contact with water. However, after 90  
278 min in water, a loss of these carbonyl bands was observed, and this was attributed to the  
279 release of all the CO from the complex (Fig. 1D).

### 280 ***EBOR-CORM-1 activity against planktonic and biofilm cells of P. aeruginosa*** 281 ***PAO1***

282 We found that applying EBOR-CORM-1 had generally negative effects on *P. aeruginosa*  
283 PAO1 growth both after 8 and 24 hours of application ( $F_{4, 25} = 50.9, p < 0.001$  and  $F_{4, 25} =$   
284  $31.8, p < 0.001$  for proportion of living cells after 8 and 24 hours, respectively, Fig. 2A) and  
285 that these negative effects increased along with the increasing concentration of applied  
286 EBOR-CORM-1 (regression analysis:  $F_{1, 24} = 43, p < 0.001$  and  $F_{1, 24} = 35, p < 0.001$  for  
287 proportion of living cells after 8 and 24 hours, respectively, Fig. 2A). Similarly, EBOR-  
288 CORM-1 was highly effective against both established planktonic and biofilm *P. aeruginosa*  
289 PAO1 cultures ( $F_{4, 25} = 77.5, p < 0.001$  and  $F_{4, 25} = 39.5, p < 0.001$ , respectively, Fig. 2A-B)  
290 and the antimicrobial activity of CORM increased in a density-dependent manner (regression  
291 analysis:  $F_{1, 24} = 92, p < 0.001$  and  $F_{1, 24} = 54, p < 0.001$ , respectively, Fig. 2A-B).

### 292 ***EBOR-CORM-1 activity against planktonic and biofilm cells of clinical P.*** 293 ***aeruginosa cystic fibrosis isolates***

294 Similar to strain PAO1, we found that EBOR-CORM-1 had inhibitory effects on all tested  
295 clinical *P. aeruginosa* isolates after 8 hours of application of CORM ( $F_{1, 152} = 11969, p <$   
296  $0.001$ , Fig. 3A). While this effect did not depend on the lineage (CORM  $\times$  lineage:  $F_{1, 152} =$   
297  $1.4, p < 0.001$ ), it varied between different clinical isolates (CORM  $\times$  isolate:  $F_{18, 152} = 11969,$   
298  $p < 0.001$ , Fig. 3A). In contrast, EBOR-CORM-1 had slightly positive effects on *P.*  
299 *aeruginosa* growth after 24 hours of application ( $F_{1, 152} = 256, p < 0.001$ , Fig. 3B) and this  
300 effect varied between different isolates (CORM  $\times$  strain:  $F_{18, 152} = 2.8, p = 0.001$ ) being  
301 slightly stronger (i.e. positive) with isolates belonging to a lineage B (CORM  $\times$  lineage:  $F_{1,$

302  $_{152} = 24.9, p < 0.001$ , Fig. 3B). EBOR-CORM-1 also had negative effects when applied to  
303 established *P. aeruginosa* cell cultures ( $F_{1, 152} = 222, p < 0.001$ , Fig. 3C). However, these  
304 effects depended on the isolate (CORM  $\times$  isolate:  $F_{18, 152} = 2.8, p = 0.001$ ) and the lineage  
305 ( $F_{1, 152} = 65.2, p = 0.001$ ), reduction being relatively larger with isolates belonging to lineage  
306 A (Fig. 3C). In the case of established biofilms, EBOR-CORM-1 had a slightly positive  
307 effect ( $F_{1, 152} = 9.6, p = 0.002$ , Fig. 3D) and while this effect varied between different isolates  
308 ( $F_{18, 152} = 2.0, p = 0.01$ ) it did not differ between the lineages ( $F_{1, 152} = 1.2, p = 0.265$ ,  
309 respectively, Fig. 3D). Together these results suggest that compared to strain PAO1, EBOR-  
310 CORM-1 effects varied more with the clinical *P. aeruginosa* isolates having negative, neutral  
311 or positive effects on bacterial growth depending on the isolate identity, lineage and the  
312 timing of CORM application.

### 313 **Linking EBOR-CORM-1 antimicrobial activity with clinical *P. aeruginosa* isolate** 314 **virulence and growth**

315 We found that all the isolates belonging to a lineage A formed non-mucoid colonies (6 out of  
316 6), while most of the isolates belonging to a lineage B formed mucoid (i.e., mucus-like)  
317 colonies (11 out of 13) on LB plates (typical mucoid and non-mucoid colonies shown in  
318 supplementary figure 3). All clinical isolates differed from the non-mucoid PAO1 strain  
319 respective of their virulence and growth (Fig. 4A). More specifically, clinical isolates  
320 produced less pyoverdine ( $F_{1, 23} = 286, p < 0.001$ ) and pyocyanin ( $F_{1, 23} = 170, p < 0.001$ ) and  
321 grew slower ( $F_{1, 23} = 91, p < 0.00$ ) and reached lower maximum densities in LB medium ( $F_{1,$   
322  $_{23} = 15.5, p = 0.001$ , Fig. 4A). However, clinical isolates produced a considerably larger  
323 amount of biofilm ( $F_{1, 23} = 21.7, p < 0.001$ ) and showed very low virulence (high time to  
324 death) in wax moth larvae *in vivo* ( $F_{1, 23} = 1296, p < 0.001$ , Fig. 4A).

325 When comparing the two CF lineages, we found that isolates belonging to a lineage B  
326 consistently outperformed the isolates belonging to a lineage A by producing more  
327 pyoverdine ( $F_{1, 18} = 6.06, p = 0.025$ ), biofilm ( $F_{1, 18} = 15.08, p = 0.001$ ) and by growing faster  
328 ( $F_{1, 18} = 22.35, p < 0.001$ ) and to higher maximum densities ( $F_{1, 18} = 6.27, p = 0.023$ ) in LB  
329 medium (Fig. 4A; Supplementary figure 4). However, lineages did not differ in pyocyanin  
330 production ( $F_{1, 18} = 1.99, p = 0.176$ ) or virulence ( $F_{1, 18} = 1.03, p = 0.324$ ; Fig. 4A;  
331 Supplementary figure 4). Across all clinical isolates, density reduction by CORM correlated  
332 negatively with biofilm formation ( $F_{1, 18} = 4.8, p = 0.042$ ). Together these results suggest that  
333 clinical isolates differed from PAO1 and from each other respective to various life-history  
334 traits important for establishing an infection.

### 335 **EBOR-CORM-1 activity against clinical *P. aeruginosa* CF isolate co-cultures**

336 Despite the observed isolate-specific variation in *P. aeruginosa* monocultures, EBOR-  
337 CORM-1 was effective in reducing the growth of *P. aeruginosa* co-cultures after 24 hours of  
338 application (CORM:  $F_{1, 24} = 132$ ,  $p < 0.001$ , Fig. 4B). Moreover, this reduction was the same  
339 regardless of whether the mix contained only one lineage or both lineages (CORM  $\times$  co-  
340 culture:  $F_{2, 24} = 0.5$ ,  $p = 0.612$ ). These results suggest that intraspecific *P. aeruginosa*  
341 population heterogeneity makes the bacteria more susceptible to EBOR-CORM-1 treatment.

#### 342 **EBOR-CORM-1 activity against *P. aeruginosa* strains in wax moth model**

343 We found that *P. aeruginosa* isolates differed in their virulence (time to death) from each  
344 other ( $F_{2, 24} = 12.2$ ,  $p < 0.001$ ): PAO1 and LESB58 strains were equally virulent, and both  
345 exhibited higher virulence than the clinical isolate 36 (killing larvae approximately in 17  
346 hours [PAO1], 36 hours [LESB58] and 92 hours [clinical isolate 36]; values averaged over  
347 both non-CORM and CORM treatments, Fig. 5). In contrast to *in vitro* results, application of  
348 EBOR-CORM-1 did not increase the survival of infected larvae ( $F_{1, 24} = 1.3$ ,  $p = 0.257$ ) with  
349 any of the infected strains (CORM  $\times$  strain:  $F_{2, 24} = 1.4$ ,  $p = 0.273$ , Fig. 5). All larvae became  
350 highly pigmented (black throughout) during the infection regardless of the *P. aeruginosa*  
351 isolate.

## 352 **4. DISCUSSION**

353 Here we set out to study the antimicrobial activity of [NEt<sub>4</sub>][MnBr<sub>2</sub>(CO)<sub>4</sub>], EBOR-CORM-1,  
354 against clinical *P. aeruginosa* isolates *in vitro*. This CORM was chosen as a suitable  
355 representative of this class of molecule based on the aqueous solubility, facile synthesis  
356 (Angelici, 1964), content of a non-toxic metal core, and simple architecture which makes it  
357 akin to a “parent compound” for CORMs that have been engineered to possess sophisticated  
358 CO release mechanisms. In contrast to more complex CORMs, the molecule was shown to  
359 have a water activated mechanism of CO release, as seen in previous studies of [MX(CO)<sub>5</sub>]  
360 species, where X is a halide (Zhang et al., 2009). Such water induced degradations are  
361 believed to proceed via a two-step pathway whereby water causes loss of the halide followed  
362 by formation of a dimer species; from which the CO is released. This may explain the  
363 changes in the IR spectra recorded in water when compared to chloroform, although the data  
364 do not directly match those for [Mn<sub>2</sub>Br<sub>2</sub>(CO)<sub>8</sub>] (El-Sayed and Kaesz, 1963), the product  
365 expect on loss of Br<sup>-</sup> from EBOR-CORM-1. We found that while EBOR-CORM-1 showed  
366 density-dependent antimicrobial activity against both planktonic and biofilm cells of the  
367 widely studied laboratory-adapted strain PAO1, these effects were more varied and weaker  
368 against clinical CF lung isolates. Regardless, EBOR-CORM-1 was efficient at reducing the

369 growth of CF isolate lineage mixes, which suggests that it could have therapeutic potential in  
370 controlling heterogeneous *P. aeruginosa* infections. Solutions of inactivated EBOR-CORM-1  
371 were essentially inactive against *P. aeruginosa* strain PAO1 (Supplementary figure 2)  
372 implying that, at least in this case, the observed activity was due to CO released from the  
373 complex rather than the residual metal salts (or indeed  $[\text{NEt}_4]^+$ ).

374 Similar to a study published by Murray *et al.* (2012), we found considerable variation in  
375 CORM antimicrobial activity between different clinical CF isolates, which depended whether  
376 we explored EBOR-CORM-1 effects on relatively short (8 hours) or long timescales (24  
377 hours) and if we compared CORM antimicrobial activity on actively growing and established  
378 cell cultures (after 48 hours of bacterial growth). Our results after 8 hours of EBOR-CORM-1  
379 application are very similar to a previous study (Murray *et al.*, 2012) showing clear reduction  
380 in bacterial densities. However, this effect vanished by the 24 hour time point, and  
381 surprisingly, some bacterial isolate cultures reached higher optical densities in the presence  
382 compared to absence of CORM, which could have been due to increase in number of cells or  
383 expression of exoproducts that were picked up by OD600 nm (e.g. pyocyanin or alginate).  
384 The most likely explanation for this is that CORM effects were short-lived (Fig. 1), which  
385 allowed bacteria to recover and grow to high densities during 24 hours after application of  
386 CORM. However, when CORM effects were measured after 4 hours of application to  
387 established cell cultures, we could still observe clear reduction in mean bacterial densities.  
388 Together these results suggest that CORM effects could be seen up to 4 hours post  
389 application and that CORM could eradicate bacterial cells whether they are at exponential or  
390 stationary phase of their growth. Interestingly, CORM effects varied between clinical isolates  
391 and were clearer with the isolates belonging to lineage A. While Murray *et al.* (2012) did not  
392 observe clear variation in CORM effects against planktonic cell cultures, they found  
393 differences in CORM efficiency in eradicating bacterial biofilms. This is also consistent with  
394 our data and reinforces the hypothesis that *P. aeruginosa* clinical isolates are likely to  
395 respond differently to CORM therapies.

396 To explore clinical isolate variation in more detail, we compared differences in bacterial  
397 virulence and growth traits between the PAO1 and clinical CF lung isolates. We found that  
398 relative to strain PAO1, clinical CF isolates grew slower, had lowered virulence and  
399 produced lower amounts of pyoverdine and pyocyanin, which are important virulence factors  
400 (O'Brien *et al.*, 2017). This is consistent with previous research and typical for *P. aeruginosa*  
401 isolates retrieved from chronic lung infections (Smith *et al.*, 2006;Folkesson *et al.*,  
402 2012;Marvig *et al.*, 2013;Williams *et al.*, 2015). The clinical isolates produced much more

403 biofilm compared to strain PAO1 and biofilm formation was the highest in the isolates  
404 belonging to lineage B. Biofilms could potentially provide a protective function against  
405 CORMs. Biofilms often have much higher antibiotic resistance than their aquatic  
406 counterparts (Stewart and William Costerton, 2001) and there are multiple reasons for this.  
407 First, antibiotics might be ineffective because the biofilm acts as a diffusion barrier (de Beer  
408 et al., 1997). Second, subpopulations within the biofilm can sometimes differentiate into a  
409 highly protected phenotypes that can repopulate the biofilms (Cochran et al., 2000). Third,  
410 the biofilm might change the chemical microenvironment, forming zones of nutrient and  
411 oxygen depletion or waste accumulation that prevents the antibiotics from functioning  
412 optimally (de Beer et al., 1994). Although we did not explore this specifically, clinical  
413 isolates belonging to a lineage A were more susceptible to CORMs and produced relatively  
414 less biofilm compared to strains belonging to a lineage B. Thus, overall a negative correlation  
415 was found between density reduction by CORM and biofilm formation. Our results therefore  
416 suggest that biofilm might provide a protective function against the CORM.

417 Despite the isolate variations observed in bacterial monocultures, EBOR-CORM-1 was  
418 effective at reducing the growth of *P. aeruginosa* clinical isolate mixed cultures. One  
419 explanation for this is that, in addition to CORM, *P. aeruginosa* growth was limited by  
420 antagonistic intraspecific species interactions in co-cultures. *P. aeruginosa* has been shown to  
421 exert both facilitative and antagonistic effects on each other via siderophore (Harrison et al.,  
422 2008) and bacteriocin (Ghoul et al., 2015) production. In our case, all the clinical isolates  
423 were derived from the same Liverpool Epidemic Strain clonal lineage and therefore likely  
424 carried the same siderophore and bacteriocin genes. Additionally, resource competition is  
425 likely to further limit *P. aeruginosa* growth both in CF lungs and simplified laboratory  
426 microcosms. As a result, even though some clinical strains were relatively insensitive to  
427 EBOR-CORM-1, their growth could have been constrained by competition with the other  
428 strains in co-cultures. We found that this was the case for all strain mixes regardless if the  
429 strains belonged to a lineage A, B or them both. This suggests that the susceptibility of the  
430 lineages measured in monocultures did not predict the susceptibility of isolate mixes within  
431 or between lineages. However, such antagonism was not observed in the absence of EBOR-  
432 CORM-1, which suggests that CORM-triggered antagonistic intraspecific interactions in *P.*  
433 *aeruginosa* co-cultures. Mechanistically, this could have been driven by competition sensing  
434 in response to CORM-mediated cell damage in *P. aeruginosa* populations (Cornforth and  
435 Foster, 2013). However, this needs to be confirmed in future experiments. Interestingly, all  
436 the clinical strains we used originated from a single CF patient and interactions between them  
437 thus reflect the realistic ecology of CF lungs. In the future, it would be useful to determine

438 pairwise interactions between these CF strains and look at CORM effects on other coexisting  
439 bacterial species observed in CF infections (Folkesson et al., 2012).

440 We found that EBOR-CORM-1 had no clear therapeutic benefits in the wax moth infection  
441 model. There are several potential explanations for this. First, EBOR-CORM-1 had limited  
442 long-term activity when in contact with water. As a result, the bactericidal effect may only  
443 have elicited lag in the initial phase of bacterial growth and proliferation within the wax  
444 moths. Second, insect tissue is not homogeneous and it is possible that we failed to deliver  
445 the CORM to the specific area of infection, or that bacteria were able to colonise new areas  
446 that were not exposed to the CORM. Third, insects differ from laboratory media (such as LB)  
447 as a bacterial growth environment, which could also affect pathogen virulence. For example,  
448 it has been recently demonstrated that plant versus animal based growth media can have  
449 physiological effects on bacterial virulence (Ketola et al., 2016) and that LB media does not  
450 adequately reflect *P. aeruginosa* growth on lung tissue (Harrison et al., 2014; Harrison and  
451 Diggle, 2016). Hence, the wax moth injection model might not reliably reflect the virulence  
452 of CF isolates derived from chronic infections. However, it is also the case that many of the  
453 affordable and available CF infection animal models do not truly reflect the real CF lung  
454 disease environment. It remains to be established whether CORM therapy could be applied in  
455 the context of CF lung infections. It is possible, for example, that it might be more suitable  
456 for treating topical infections such as burn wounds, for which better animal models are  
457 available (Rumbaugh et al., 2012).

458 Further work is also needed to understand the mode of action of EBOR-CORM-1. While  
459 respiratory oxidases and globins at heme targets are generally considered the prime targets of  
460 CO and CORMs (Wareham et al., 2015), it has been demonstrated that CORMs can have  
461 multiple different other targets (Wilson et al., 2015). For example, CO also binds to the di-  
462 iron site in bacterial NO reductases and to iron, copper, and nickel sites in certain microbial  
463 proteins such as CO dehydrogenase (Lu et al., 2004; Wasser et al., 2005). In some cases,  
464 CORMs might have intracellular targets but their accumulation within the cells can be very  
465 weak (Tinajero-Trejo et al., 2016). Moreover, in the future it would be important to test if  
466 EBOR-CORM-1 is cytotoxic to eukaryotic cells. The concentration we used are in line with  
467 previously published work where no, or very mild, cytotoxic effects were observed (Murray  
468 et al., 2012). We are currently conducting experiments to validate this independently and to  
469 understand how EBOR-CORM-1 interacts with bacterial cells. While, our wax moth assays  
470 show that the concentrations we used had no negative effects on short-term insect viability,  
471 more detailed cytotoxicity assays are needed in the future. Lastly, the low solubility of

472 EBOR-CORM-1 in water, and its activation in this medium, is problematic for delivery and  
473 activation at specific sites within patients. In addition to chemically increasing the molecule  
474 stability, CORMs could be enclosed in microvesicles (van Dommelen et al., 2012) to ensure  
475 more efficient antimicrobial activity and drug delivery.

476 In conclusion, our results show that EBOR-CORM-1 shows antimicrobial activity against  
477 both planktonic and biofilm cells of *P. aeruginosa* strain PAO1 but that these effects are  
478 more varied and less pronounced against clinical CF lung isolates in monocultures. In  
479 contrast, more heterogeneous *P. aeruginosa* populations comprising intraspecific phenotypic  
480 variants were more susceptible to CORM treatment. This potentially has wider implications  
481 in the testing of novel therapeutics. At present, this is done almost exclusively using clonal *P.*  
482 *aeruginosa* populations. Our observations suggest that testing carried out on more  
483 heterogeneous populations of *P. aeruginosa*, more closely resembling those found in the CF  
484 lung, may give different and sometimes more promising results.

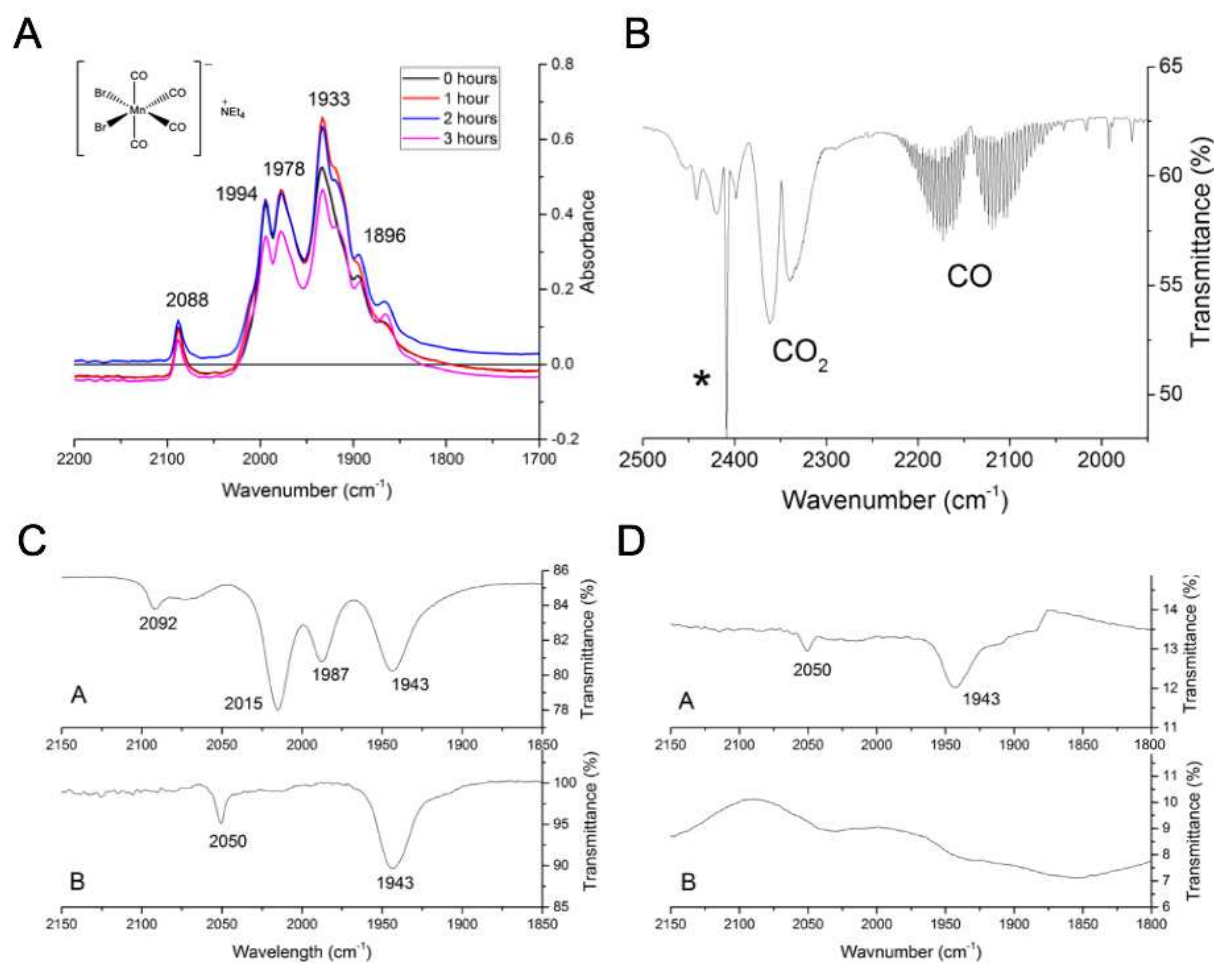
485

## 486 **ACKNOWLEDGEMENTS**

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491

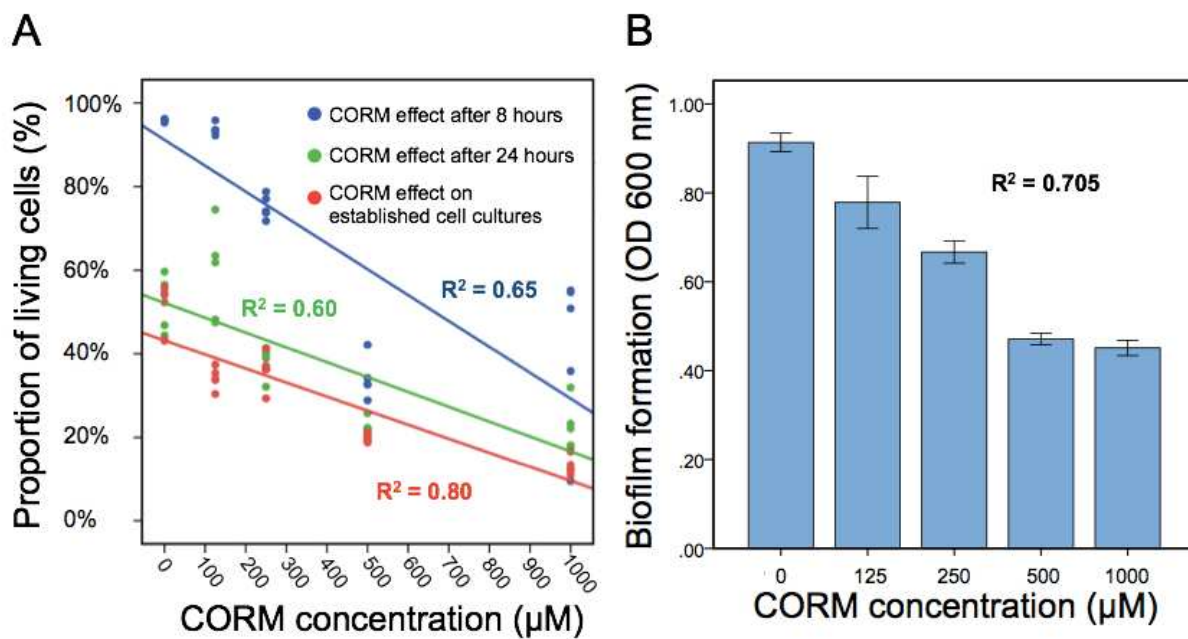
## 492 **FIGURES AND FIGURE LEGENDS**



493

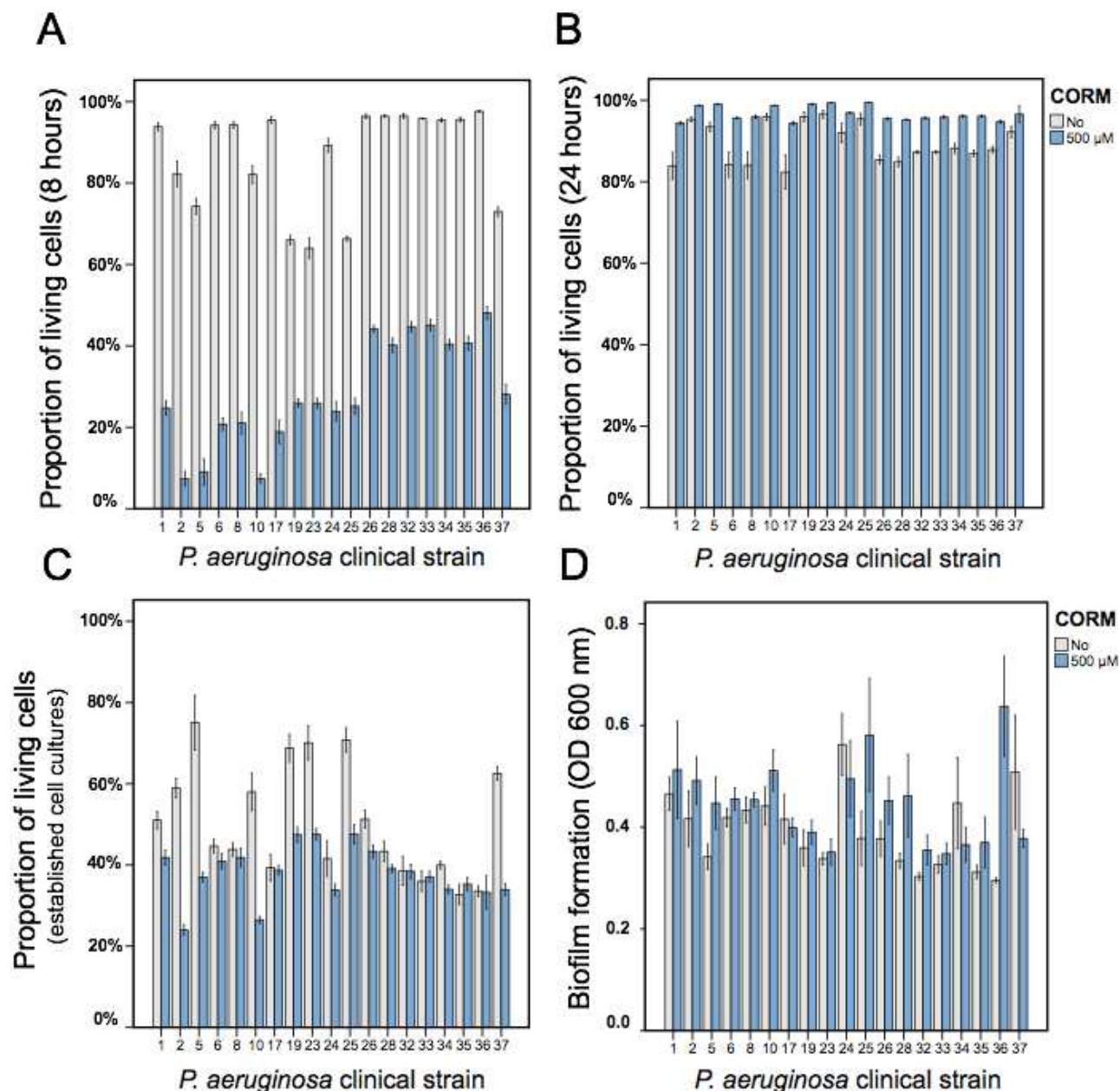
494 **Figure 1.** The stability of EBOR-CORM-1. Panel A shows the IR spectra of EBOR-CORM-  
 495 1 upon heating at 50° C for 0 hours (black line), for 1 hours (red line), for 2 hours (blue) and  
 496 for 3 hours (pink). The structure of the EBOR-CORM-1 is shown in inset on the left. Panel B  
 497 shows the gas phase IR spectra of EBOR-CORM-1 in chloroform with added water where  
 498 the \* indicates a band from chloroform. Panel C shows the IR spectra of EBOR-CORM-1 in  
 499 chloroform (top) and water (bottom) and panel D the IR spectra of CO in water after 1 min  
 500 (top) and 90 min (bottom) dissolution. All frequencies given are in  $\text{cm}^{-1}$ .

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502

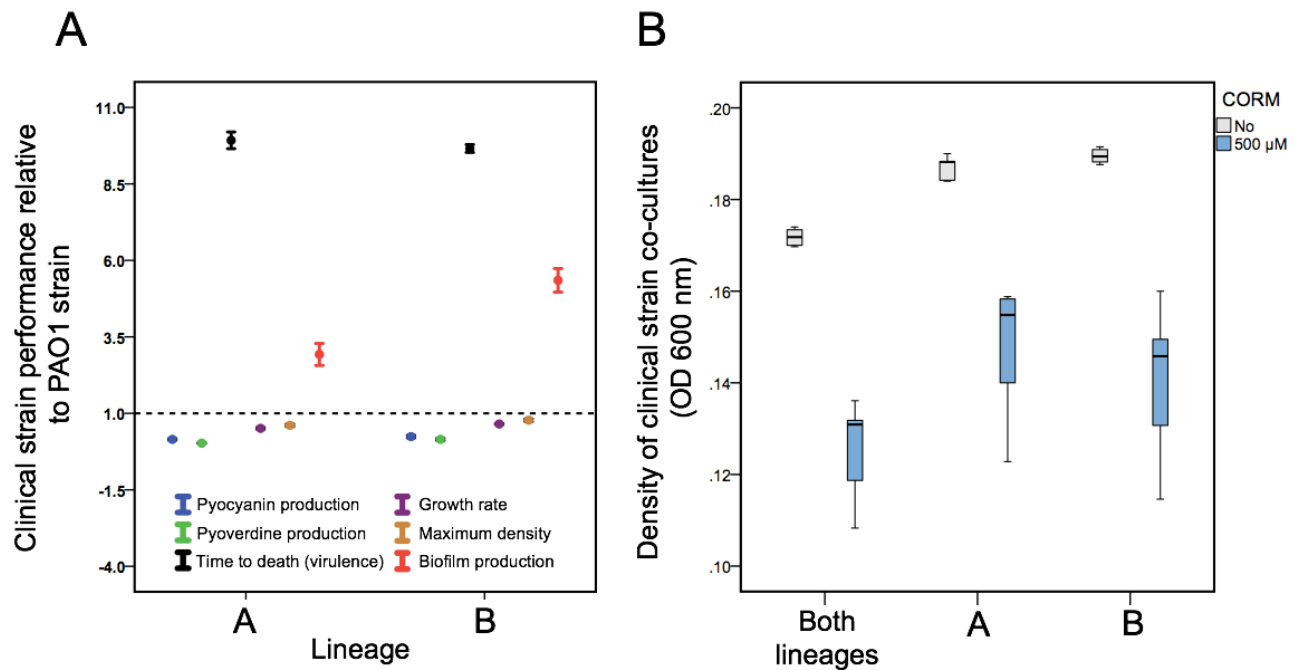
503 **Figure 2.** The EBOR-CORM-1 effects on planktonic and biofilm cells of *P. aeruginosa*  
504 PAO1. In panel A, different lines denote for cell densities after 8 hours (blue line) and 24  
505 hours (green line) of EBOR-CORM-1 application and EBOR-CORM-1 effects on established  
506 cell cultures (red line) in different CORM concentrations. Panel B shows EBOR-CORM-1  
507 effects on PAO1 biofilms in different CORM concentrations. The  $R^2$  denotes for the fit of  
508 regression with our data, and in panel B, bars denote for  $\pm 1$  s.e.m.



510

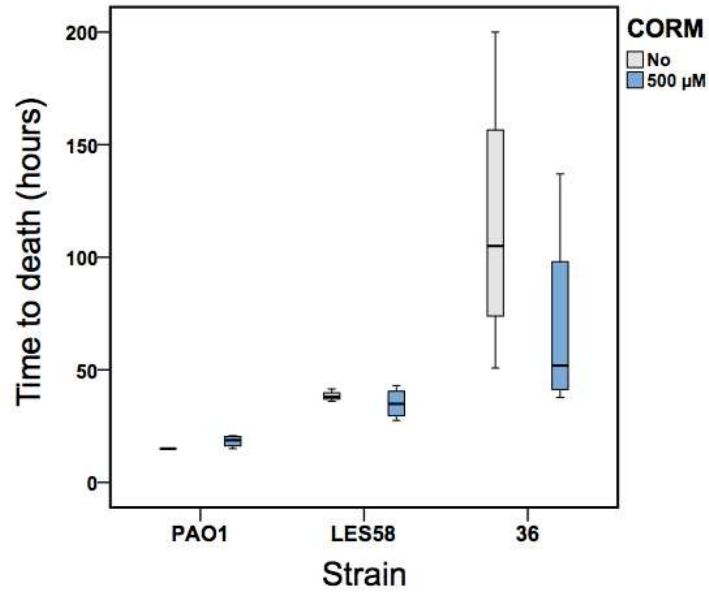
511 **Figure 3.** The EBOR-CORM-1 effects on planktonic and biofilm cells of clinical *P.*  
 512 *aeruginosa* CF isolates. Panels A and B show the proportion of living cells after 8 hours and  
 513 24 hours of EBOR-CORM-1 application, respectively. Panel C and D show the EBOR-  
 514 CORM-1 effects on established cell cultures and biofilms, respectively. In all panels, bars  
 515 denote for  $\pm 1$  s.e.m.

516



517  
518

519 **Figure 4.** Differences in *P. aeruginosa* growth and virulence trait variation between PAO1  
 520 and clinical CF isolates (panel A) and EBOR-CORM-1 effects on clinical CF isolate lineage  
 521 mixes (panel B). In panel A, different colours denote for pyocyanin (blue) and pyoverdine  
 522 (green) production, time to death (black), growth rate (purple), maximum density (yellow)  
 523 and biofilm production for clinical isolates belonging to lineages A and B. The dashed line  
 524 shows the mean performance of PAO1 strain. Panel B shows EBOR-CORM-1 effect on  
 525 clinical CF isolate mixes after 24 hours of CORM application. In panel A, bars denote for  $\pm 1$   
 526 s.e.m., and in panel B, extreme values around lower and upper quartile (black line shows the  
 527 median).



528

529 **Figure 5.** The EBOR-CORM-1 activity against three *P. aeruginosa* strains in wax moth  
 530 model. Boxplots show larval survival in the absence (light grey) and presence (blue) of  
 531 EBOR-CORM-1 for PAO1, LESB58 and clinical isolate #36 (lineage B). Bars show extreme  
 532 values around lower and upper quartile and black lines show the median.

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