

Evolution of Colistin Resistance in the *Klebsiella pneumoniae* Complex Follows Multiple Evolutionary Trajectories with Variable Effects on Fitness and Virulence Characteristics

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1 **Evolution of colistin resistance in the *Klebsiella pneumoniae* complex follows multiple evolutionary**
2 **trajectories with variable effects on fitness and virulence characteristics.**

3

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13

14 Running title: colistin resistance in *Klebsiella*

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16

17

18

19 **Abstract**

20 The increasing prevalence of multidrug-resistant *Klebsiella pneumoniae* has led to a resurgence in
21 the use of colistin as last-resort drug. Colistin is a cationic antibiotic that selectively acts on Gram-negative
22 bacteria through electrostatic interactions with anionic phosphate groups of the lipid A moiety of
23 lipopolysaccharides (LPS). Colistin resistance in *K. pneumoniae* is mediated through loss of these
24 phosphate groups, their modification by cationic groups, and by the hydroxylation of acyl-groups of lipid
25 A. Here, we study the *in vitro* evolutionary trajectories towards colistin resistance in four clinical
26 *K. pneumoniae* complex strains and their impact on fitness and virulence characteristics.

27 Through population sequencing during *in vitro* evolution, we found that colistin resistance
28 develops through a combination of single nucleotide polymorphisms, insertion and deletions, and the
29 integration of insertion sequence elements, affecting genes associated with LPS biosynthesis and
30 modification, and capsule structures. Colistin resistance decreased the maximum growth rate of one
31 *K. pneumoniae sensu stricto* strain, but not in the other three *K. pneumoniae* complex strains.
32 Colistin-resistant strains had lipid A modified through hydroxylation, palmitoylation, and L-Ara4N
33 addition. *K. pneumoniae sensu stricto* strains exhibited cross-resistance to LL-37, in contrast to the
34 *K. variicola* subsp. *variicola* strain. Virulence, as determined in a *Caenorhabditis elegans* survival assay,
35 was increased in two colistin-resistant strains.

36 Our study suggests that nosocomial *K. pneumoniae* complex strains can rapidly develop colistin
37 resistance through diverse evolutionary trajectories upon exposure to colistin. This effectively shortens the
38 lifespan of this last-resort antibiotic for the treatment of infections with multidrug-resistant *Klebsiella*.

39

40 **Introduction**

41 *Klebsiella pneumoniae* is a Gram-negative opportunistic pathogen and a leading cause of
42 hospital-associated infections such as pneumonia, surgical site infections, and urinary tract infections.
43 *K. pneumoniae* may also asymptotically colonize the skin, upper respiratory tract, and digestive tract of
44 healthy individuals (1, 2). The *K. pneumoniae* complex is genetically diverse, with different phylogroups
45 within the complex corresponding to different species and sub-species, each occupying specific niches (1,
46 2). The *K. pneumoniae sensu stricto* and *K. quasipneumoniae* phylogroups are associated with human
47 intestinal carriage, whilst the *K. variicola* phylogroup is associated with plants and bovines (1, 3). Of all
48 strains isolated from human infections and typed as *K. pneumoniae*, the majority is *K. pneumoniae sensu*
49 *stricto*, but *K. variicola* and *K. quasipneumoniae* have also been found to cause infections in patients and
50 are frequently misidentified as *K. pneumoniae* (4, 5). Although infections with strains from the *K. variicola*
51 phylogroup are relatively rare, they have been associated with the highest mortality rate within the
52 *K. pneumoniae* complex (3).

53 In recent years, *K. pneumoniae* complex strains have rapidly emerged as multidrug-resistant
54 pathogens through acquisition of resistance to third-generation cephalosporins, fluoroquinolones, and
55 aminoglycosides, and have increasingly become resistant to carbapenems through the acquisition of
56 carbapenemases (6–9). The increasing prevalence of multidrug resistance within the *K. pneumoniae*
57 complex, and the lack of development of novel antibiotic classes effective against Gram-negative bacteria,
58 have limited the available therapeutic options against multidrug-resistant *K. pneumoniae* complex strains.
59 These limitations have prompted the resurgence in the use of the antibiotic colistin in treatment of infections
60 by *K. pneumoniae* complex strains (10–12). After its introduction into clinical practice in the 1950s, colistin
61 fell into disuse in human medicine in the 1970s because of the neuro- and nephrotoxic side effects
62 associated with its use and the development of safer classes of antibiotics. Due to the emergence

63 multidrug-resistant Gram-negative opportunistic pathogens, like *K. pneumoniae*, it has recently regained
64 clinical relevance as a last-line antibiotic (13).

65 Colistin (polymyxin E) is a cationic, amphipathic molecule composed of a fatty acid chain linked
66 to a non-ribosomally synthesized decapeptide (14, 15). The mechanism of action of colistin relies on the
67 selective presence of the negatively charged lipopolysaccharides (LPS) in the membranes of Gram-negative
68 bacteria. The negative charges of LPS are carried by the anionic phosphate groups of the lipid A moiety of
69 LPS, which enable colistin to bind through electrostatic interactions (14). Insertion of colistin into the outer
70 membrane leads to membrane permeabilization. The subsequent destabilization of the cytoplasmic
71 membrane, where LPS is present after synthesis in the cytoplasm while awaiting transport to the outer
72 membrane, ultimately leads to cell death (14, 16, 17).

73 The increased use of colistin to treat infections with multidrug-resistant Gram-negative bacteria,
74 especially in low- and middle-income countries (12), and the use of colistin in livestock farming, either
75 therapeutically to treat enteric infections or as a growth promoter (18), has led to a rise in colistin resistance
76 in *K. pneumoniae* from clinical, veterinary, and environmental sources (9, 18, 19). Colistin resistance in
77 *K. pneumoniae* complex strains is mostly mediated through decoration of lipid A with cationic groups, to
78 counteract the electrostatic interactions between colistin and lipid A (14). These modifications can be the
79 result of point mutations and insertion-deletions (indels) in chromosomally located genes (including
80 *phoPQ*, *pmrAB*, and *crrAB*) resulting in amino acid substitutions, insertions, and deletions in the proteins
81 encoded by these genes (20–23). In addition, the acquisition of mobile genetic elements carrying a member
82 of the *mcr*-gene family may also lead to lipid A modification (23, 24). In *K. pneumoniae*, the inactivation
83 of *mgrB* encoding a negative regulator of the two-component regulatory system PhoPQ, through the
84 insertion of an insertion sequence (IS) element, or a mutation leading to the formation of a premature stop
85 codon, is a particularly frequently observed colistin resistance mechanism (25–29). Other mechanisms of

86 colistin resistance in *K. pneumoniae* include the upregulated expression of efflux pumps (30, 31), changes
87 in LPS production (20, 32), and the overproduction of capsular polysaccharides (33, 34).

88 Upon infection the innate immune system will attempt to neutralize invading bacteria. The cellular
89 components of the innate immune system can detect Gram-negative bacteria through the presence of LPS
90 (35). Activated immune cells can kill bacteria and will attempt to kill them by unleashing bactericidal
91 components including the antimicrobial peptide LL-37. Similar to colistin, LL-37 relies on electrostatic
92 interactions with LPS for its mechanism of action (36). Modifications to LPS may influence the efficacy of
93 bactericidal components, and may thus result in altered virulence by reducing the effectiveness of these
94 components (35–37). Modifications capable of affecting the efficiency of the immune system include
95 neutralization of the anionic charges carried by lipid A, and changes in acylation of lipid A (35, 38, 39).
96 These changes are mediated through the PhoPQ and PmrAB two-component regulatory systems. Notably,
97 colistin resistance is mediated through the same modifications and two-component regulatory systems. The
98 development of colistin resistance may thus also affect virulence characteristics.

99 To better understand the mechanisms and consequences of colistin resistance in *K. pneumoniae*
100 complex strains, we determined the evolutionary trajectories of three *K. pneumoniae sensu stricto* strains
101 and one *K. variicola* subsp. *variicola* strain towards colistin resistance in an *in vitro* evolution experiment,
102 and determined how colistin resistance impacted fitness, LPS modifications, and virulence characteristics.

103

104 **Materials and Methods**

105

106 **Ethical statement**

107 The colistin-susceptible *K. pneumoniae* complex strains used in this study were isolated as part of
108 routine diagnostic procedures, which did not require consent or ethical approval by an institutional review
109 board.

110

111 **Bacterial strains, growth conditions, and chemicals**

112 The colistin-susceptible KP209, KP040, KP257, and KV402 strains were retrospectively, obtained
113 from the diagnostic laboratory of the University Medical Center Utrecht in Utrecht, the Netherlands. In
114 initial routine diagnostic procedures, they were identified as *K. pneumoniae sensu stricto* by matrix-assisted
115 laser desorption–ionisation time-of-flight (MALDI-TOF) on a Bruker microflex system (Leiderdorp, The
116 Netherlands). Colistin susceptibility testing of the clinical isolates was initially performed on a BD Phoenix
117 automated identification and susceptibility testing system (Becton Dickinson, Vianen, The Netherlands).
118 All strains were grown either in lysogeny broth (LB; Oxoid, Landsmeer, The Netherlands) with agitation
119 at 300 rpm, or on LB agar, at 37°C, unless otherwise specified. Colistin sulphate was obtained from Duchefa
120 Biochemie (Haarlem, The Netherlands).

121

122 **Determination of minimal inhibitory concentration of colistin**

123 Minimal inhibitory concentrations (MICs) to colistin were determined as described previously (40)
124 in line with the recommendations from the joint Clinical & Laboratory Standards Institute and European

125 Committee on Antimicrobial Susceptibility Testing (EUCAST) Polymyxin Breakpoints Working Group
126 ([http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf)
127 [for MIC determination of colistin March 2016.pdf](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf)). In short, colistin susceptibility testing was
128 performed using BBL™ Mueller Hinton II (cation-adjusted) broth (MHCAB; Becton Dickinson), untreated
129 Nunc 96-wells round bottom polystyrene plates (Thermo Fisher Scientific, Landsmeer, The Netherlands),
130 and Breathe-Easy sealing membranes (Sigma-Aldrich, Zwijndrecht, The Netherlands). The MIC was
131 observed after stationary, overnight growth at 37°C, and was determined to be the lowest concentration
132 where no visible growth was observed. The breakpoint value for colistin resistance of an MIC > 2 µg/ml
133 was obtained from EUCAST (http://www.eucast.org/clinical_breakpoints/).

134

135 ***In vitro* evolution of colistin resistance**

136 The nosocomial, colistin-susceptible *K. pneumoniae* strains were evolved towards colistin
137 resistance by culturing in increasing colistin concentrations over a period of 5-7 days. As we used LB as
138 medium for the *in vitro* evolution, we first determined the colistin MICs in this medium (as outlined above)
139 and subsequently grew each strain in 1 ml LB with initial colistin concentrations of 1- and 2-times the MIC.
140 After overnight growth, 1 µl of the cultures with the highest concentration of colistin that had visible growth
141 were used to propagate a fresh culture by inoculating 1 ml of fresh LB, supplemented with the same or
142 twice the concentration of colistin in which growth was observed in the previous day's culture
143 (Supplemental Figure S1). This process was repeated for 5-7 days. Each overnight culture was stored
144 at -80°C in 20% glycerol.

145

146 **Genomic DNA isolation and whole-genome sequencing**

147 Genomic DNA was isolated using the Wizard Genomic DNA purification kit (Promega, Leiden,
148 The Netherlands) according to the manufacturer's instructions. DNA concentrations were measured with
149 the Qubit 2.0 fluorometer and the Qubit dsDNA Broad Range Assay kit (Life Technologies, Bleiswijk, The
150 Netherlands).

151 Illumina sequence libraries of genomic DNA were prepared using the Nextera XT kit (Illumina,
152 San Diego, CA) according to the manufacturer's instructions, and sequenced on an Illumina MiSeq system
153 with a 500-cycle (2×250 bp) MiSeq v2 reagent kit (Illumina). MinION library preparation for barcoded
154 2D long-read sequencing was performed using the SQK-LSK208 kit (Oxford Nanopore Technologies,
155 Oxford, England, United Kingdom), according to the manufacturer's instructions, with G-tube (Covaris,
156 Woburn, Massachusetts, United States of America) shearing of chromosomal DNA for 2 x 120 seconds at
157 1500 g. The libraries were sequenced on a MinION sequencer (Oxford Nanopore Technologies) through a
158 SpotON Flow Cell Mk I (R9.4; Oxford Nanopore Technologies).

159

160 **Genome assembly and annotation**

161 The quality of the Illumina sequencing data was assessed using FastQC v0.11.5
162 (<https://github.com/s-andrews/FastQC>). Illumina sequencing reads were trimmed for quality using nelsoni
163 v0.115 (<https://github.com/Victorian-Bioinformatics-Consortium/nelsoni>) using standard settings with the
164 exception of a minimum read length of 100 nucleotides. MinION reads in FastQ format were extracted
165 from Metrichor base-called FAST5-files using Poretools (41). *De novo* hybrid genome assembly of the
166 colistin-susceptible strains was performed with Illumina and Oxford Nanopore data as described previously
167 (42). Genome annotation was performed using Prokka (43).

168

169 **Phylogenetic analysis, MLST typing, and identification of antibiotic resistance genes**

170 To generate a core genome phylogeny, Illumina/Oxford Nanopore hybrid genome assemblies were
171 aligned using ParSNP v1.2 (37) with 37 publicly available *Klebsiella pneumoniae* complex genomes that
172 cover all phylogroups of the *K. pneumoniae* complex (2). To include the genome of *K. africanensis* strain
173 38679, we assembled the genome from raw reads, by processing the raw sequence reads using Nasoni with
174 standard settings, except for minimum read length (75 nucleotides), and subsequent assembly by SPAdes
175 with kmers 21, 33, 55, 77 and the “careful” options turned on.

176 Figtree was used to visualize and midpoint root the phylogenetic tree (<http://tree.bio.ed.ac.uk/>).
177 MLST typing was performed using the mlst package v2.10 (<https://github.com/tseemann/mlst>). Genome
178 assemblies of colistin-susceptible strains were assessed for antibiotic resistance genes by ResFinder 3.1
179 through standard settings (44).

180

181 **Determination of SNPs and indels between axenic colistin-susceptible and colistin-resistant strain**
182 **pairs.**

183 Read-mapping of Nasoni-filtered reads of evolved strains to the genomes of the isogenic
184 colistin-susceptible parental strains was performed using Bowtie2 (45). SNP and indel-calling was
185 performed using SAMtools 0.1.18 using the following settings: Qscore ≥ 50 , mapping quality ≥ 30 , a
186 mapping depth ≥ 10 reads, a consensus of $\geq 75\%$ to support a call, and ≥ 1 read in each direction supporting
187 a mutation, as previously described (46). To correct for potential assembly errors, we also performed the
188 SNP and indel-calling procedure by mapping the reads of the reference isolates against their own
189 assemblies. SNPs and indels found in the reference-versus-reference comparison were ignored in query-
190 versus-reference comparisons. Synonymous mutations were excluded from further analyses. SNPs and
191 indels were manually linked to genes in the assembly.

192

193 **Determination of location of IS elements in genomes**

194 To determine which IS elements were present in the genomes of colistin-susceptible strains, we
195 analysed the Illumina/Oxford Nanopore hybrid genome assemblies using ISfinder (47). Per genome, the IS
196 elements with an E-value $< 1e-50$ were selected for further study. If multiple distinct IS elements were
197 called at the same position, the element with the highest sequence identity was selected to represent that
198 position.

199 To detect changes in the position of the identified IS elements, we analysed the genomic assemblies
200 of the isogenic colistin-susceptible and colistin-resistant strain pairs through ISMapper (48). To maximize
201 the ability of ISMapper to detect IS elements in our sequencing data, the obtained nucleotide sequences of
202 the IS elements in the genome were used as input, and the --cutoff flag of ISMapper was set to 1, whilst
203 other settings remained unchanged. The results were inspected for IS elements that had different positions
204 between the colistin-susceptible, and colistin-resistant strains. Insertion of IS elements was confirmed
205 through PCRs, using DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) and primers spanning
206 the IS insertion site (Supplemental Table S1) and subsequent Sanger sequencing of the PCR product by
207 Macrogen (Amsterdam, The Netherlands).

208

209 **SNP and indel calling in evolving populations.**

210 To track the genomic changes within the growing cultures under the selective pressure of increasing
211 colistin concentrations, genomic DNA was isolated from the 5-7 overnight cultures of each *in vitro*
212 evolution experiment and sequenced on the Illumina MiSeq platform as described above. SNPs and indels
213 were called as before, with each call supported by at least 25% of reads. Once identified in one or more

214 populations, the abundance of the specific SNPs and indels were then quantified manually for all individual
215 populations of the *in vitro* evolution experiment. Mutations called within 150 bp of a contig end were
216 filtered out, as previously recommended (49). Identified SNPs and indels were manually linked to genes in
217 the genome assembly, and inspected for synonymous versus non-synonymous mutations. Non-coding
218 mutations were included in subsequent analyses, while synonymous mutations were excluded.

219

220 **Determination of growth rate**

221 To determine the maximum specific growth rate, a Bioscreen C instrument (Oy Growth Curves
222 AB, Helsinki, Finland) was used. Overnight cultures were used to inoculate 200 µl fresh LB medium
223 1:1000. Incubation was set at 37°C with continuous shaking. Growth was observed by measuring the
224 absorbance at 600 nm every 7.5 minutes. Each experiment was performed in triplicate.

225

226 **MALDI-TOF analysis of lipid A structures**

227 Isolation of lipid A molecules and subsequent analysis by negative-ion matrix-assisted laser
228 desorption–ionisation time-of-flight (MALDI-TOF) mass spectrometry was performed as previously
229 described (29, 50, 51). Briefly, *K. pneumoniae* strains were grown in LB (Oxoid) and the lipid A was
230 purified from stationary cultures using the ammonium hydroxide/isobutyric acid isolation method described
231 earlier (52). Mass spectrometry analysis were performed on a Bruker autoflex® speed TOF/TOF mass
232 spectrometer in negative reflective mode with delayed extraction using as matrix an equal volume of
233 dihydroxybenzoic acid matrix (Sigma-Aldrich) dissolved in (1:2) acetonitrile-0.1% trifluoroacetic acid. The
234 ion-accelerating voltage was set at 20 kV. Each spectrum was an average of 300 shots. A peptide calibration
235 standard (Bruker) was used to calibrate the MALDI-TOF. Further calibration for lipid A analysis was

236 performed externally using lipid A extracted from *Escherichia coli* strain MG1655 grown in LB medium
237 at 37°C.

238

239 **LL-37 survival assay**

240 In order to test the susceptibility of the *K. pneumoniae* strains to LL-37, we adapted previously
241 described protocols (53). An overnight broth culture was diluted to a concentration of 2.5×10^6 CFU/ml in
242 25% LB and incubated with or without the addition of 50 µg/ml LL-37 (AnaSpec Inc, Ferment, California,
243 United States of America) for 90 minutes at 37°C with agitation at 300 rpm in sterile round-bottom 96-well
244 plates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). After incubation, samples were serially
245 diluted in PBS and plated on LB agar plates. CFUs were counted after overnight incubation at 37°C.

246

247 ***Caenorhabditis elegans* virulence assays**

248 *Caenorhabditis elegans* strain CF512 (*rrf-3(b26) II*; *fem-1(hc17) IV*), which has a
249 temperature-sensitive reproduction defect, was obtained from the Caenorhabditis Genetics Center at the
250 University of Minnesota, Twin Cities (<http://www.cgc.cbs.umn.edu/>). CF512 nematodes were maintained
251 at 20°C on Nematode Growth Medium (NGM) agar plates seeded with *E. coli* OP50 (54), and placed on
252 fresh plates at least once per week. For seeding of NGM plates, mid-exponential phase cultures were used.
253 After reaching mid-exponential phase, the cells were washed with PBS, and 1×10^6 CFU were spread on
254 NGM plates, after which the bacterial lawns were grown overnight at 37°C.

255 To quantify bacterial virulence, *C. elegans* CF512 lifespan assays were performed with
256 synchronized nematodes according to a previously described protocol (55). For synchronization, nematodes
257 and eggs were collected from a NGM plate in ice-cold filter-sterilized M9 medium, and washed by spinning

258 at 1500 x g for 30 seconds (56). Nematodes were destructed by vigorous vortexing in hypochlorite solution
259 (25 mM NaOH, 1.28% sodium hypochlorite) for two minutes, after which the reaction was stopped by the
260 addition of M9 medium. Eggs were allowed to hatch on NGM plates seeded with *E. coli* OP50 for 6-8 hours
261 at 20°C, after which they were placed at 25°C to avoid progeny. After 48 hours, L3-L4 nematodes were
262 placed on NGM plates (n=40 per plate) seeded with bacterial strains. Plates were scored for live nematodes.
263 Nematodes were considered dead when they did not show spontaneous movement or a response to external
264 stimuli.

265

266 **Statistical analysis**

267 Statistical analyses were performed using the parametric one-way ANOVA test with a Dunnett's
268 test for multiple comparisons (for the determination of maximum growth rates), the non-parametric
269 Mann-Whitney test was used (for the LL-37 survival assay), and the Mantel-Cox log-rank test (for the *C.*
270 *elegans* assays). Statistical significance was defined as a p-value < 0.05 for all tests. Statistical analyses
271 were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, California, United
272 States of America).

273

274 **Data availability**

275 Sequence data of both the Illumina short-read, and the Oxford Nanopore long-read sequencing has
276 been deposited in the European Nucleotide Archive (accession number PRJEB29521).

277

278 **Results**

279

280 **The colistin-susceptible *K. pneumoniae* complex strains have a diverse genetic background**

281 The four clinical isolates used in this study were obtained from pus, faecal, or urine samples through
282 routine diagnostic procedures in September 2013. All four strains were initially typed as *K. pneumoniae*
283 *sensu stricto* through routine diagnostic procedures using MALDI-TOF. The susceptibility to colistin of
284 these strains, previously determined in routine diagnostic procedures, was confirmed through antibiotic
285 susceptibility testing using broth microdilution (Figure 1A).

286 The sequenced genomes of the colistin-susceptible strains were screened for acquired antibiotic
287 resistance genes through ResFinder 3.1 (Figure 1B). None of the nosocomial strains was determined to
288 carry one of the *mcr*-genes. Between two and five acquired antibiotic resistance genes were observed in the
289 genome assemblies, encoding resistance to beta-lactams, quinolones, and fosfomycin.

290 To accurately identify the phylogenetic position of these nosocomial strains within the
291 *K. pneumoniae* complex, a phylogenetic tree was generated based on the Illumina/Oxford Nanopore hybrid
292 genome assemblies of the colistin-susceptible strains, and 37 publicly available genomes covering all
293 phylogroups in the *K. pneumoniae* complex (2). Based on a 1.3 Mbp core-genome alignment, the
294 phylogenetic tree showed that strains KP209, KP040, and KP257 clustered in the *K. pneumoniae sensu*
295 *stricto* (KpI) phylogroup (Figure 1C). Strain KV402 clustered in the *K. variicola* subsp. *variicola* (KpIII)
296 phylogroup, even though it had been typed as *K. pneumoniae sensu stricto* through MALDI-TOF during
297 initial routine diagnostic procedures.

298

299 **Colistin resistance emerges through multiple evolutionary trajectories in the *K. pneumoniae* complex**

300 To understand the evolutionary trajectories through which the *K. pneumoniae* complex strains
301 evolved resistance towards colistin, we deep-sequenced each overnight culture during growth in increasing
302 concentrations of colistin (Supplemental Table S2), and identified SNP, indels and excision/integration
303 events of IS elements.

304 We observed the rapid emergence and fixation of several mutations (Figure 2) in the presence of
305 colistin. In three populations (KP209, KP257, and KV402), these mutations occurred in the genes encoding
306 the PhoPQ two-component regulatory system after one day of culturing (Supplemental Table S3). In the
307 KP040 population, we observed the integration of an IS5 element (Supplemental Table S4, Supplemental
308 Data 1) in the promoter region of both the *crrAB* operon and the divergently transcribed *crrC* gene. In
309 addition, an intergenic SNP (located in promoter regions of *ecpR* or *phnC*) in KP040 became fixed in the
310 population on the first day of culturing. Both EcpR and PhnC have not previously been associated with
311 colistin resistance. Although other mutations, in other locations, also occurred during the first day of
312 culturing, these mutations failed to become fixed in the population, and were either lost on subsequent days,
313 or did not change in abundance over time.

314 On subsequent days of the *in vitro* evolution experiment, novel mutations in the populations were
315 associated with additional increases in MIC of colistin. New SNPs that were fixed in the populations were
316 observed in *phoQ* (KP209 (day 5), and KV402 (day 6)), and *pmrB* (KP209 (day4)). In KP257, a SNP in
317 *lptD* was first observed on day 3, and was then fixed in the population. The *lptD* gene encodes a
318 barrel-shaped transporter that transports LPS onto the outer leaflet of the outer membrane (57). Mutations
319 in genes located in the capsule synthesis locus (K-locus) were also detected. In KV402 a 13 bp deletion
320 was observed in *wcaJ* from day 3 onwards, leading to a premature stop-codon. In KP040 a new insertion
321 of IS102, inactivating *wzc* was observed from day 4. In addition, a 12 bp insertion in the gene encoding the
322 Rho transcription termination factor was observed in KP040. We did not observe any mutations in the *mgrB*
323 gene in these *in vitro* evolution experiments.

324

325 ***K. pneumoniae* can rapidly develop colistin resistance without loss of fitness.**

326 To enable a further characterisation of the impact of the evolution of colistin resistance on fitness
327 and virulence characteristics, we isolated a single, random colony on non-selective medium from each day
328 of the *in vitro* evolution experiments. The genome sequences of the axenic strains of the last day of the *in*
329 *vitro* evolution experiments were determined by Illumina sequencing. SNPs, indels and IS element
330 insertions were identified in these strains in comparison with the colistin-susceptible parental strain. After
331 combining these data with the population sequencing data described above, we determined the presence of
332 these mutations in the axenic strains isolated after each day of the *in vitro* evolution experiment by targeted
333 PCRs and Sanger sequencing of the amplicons. We were thus able to correlate the occurrence of mutations
334 with increases in the MIC of colistin (determined in MHCAB) in each strain.

335 All four strains developed levels of resistance to colistin above the breakpoint value (2 µg/ml) after
336 one overnight incubation of the colistin-susceptible (MIC ≤2 µg/ml) strain in the presence of the antibiotic
337 (Figure 2). The initial mutations in *phoPQ* were associated with an increase in MIC in strains KP209 (32
338 µg/ml), KP257 (128 µg/ml), and KV402 (32 µg/ml) (Figure 2). The integration of the IS5 element in the
339 promoter region of *crrAB* and *crrC*, and the appearance of an intergenic SNP between *ecpR* and *phnC*, also
340 occur simultaneously with an increase in the MIC of colistin (4 µg/ml). The additional SNP in *phoQ* in
341 KP209 was not associated with an increase in the MIC of colistin (256 µg/ml). Integration of IS102 in *wzc*
342 of the K-locus, as well as the 12-bp insertion in the gene encoding the transcription termination factor Rho,
343 was associated with an additional increase in the MIC of colistin (128 µg/ml) in strain KP040. The SNP in
344 *lptD* in strain KP257 did not lead to a meaningful increase in the MIC of colistin (256 µg/ml). The culture
345 isolated from the last day of the KV402 *in vitro* evolution experiments had a SNP in *yciM* (Supplemental
346 Table S5), encoding a negative regulator of LPS biosynthesis (20), but this did not contribute to a further

347 reduced susceptibility to colistin. Because of the random nature of picking single isolates from their
348 populations, some mutations identified by population sequencing were not recapitulated in the axenic
349 strains and *vice versa* (Fig. 2, Supplemental Table S5).

350 The measurement of the maximum growth rate as a proxy for general fitness of the axenic strains
351 isolated on the different days of the *in vitro* evolution experiment showed that the increase in MIC of colistin
352 to values above 2 µg/ml after one overnight incubation, did not negatively affect the maximum growth rate
353 for strains KP209, KP040, and KV402. Only the initial increase in MIC of colistin in strain KP257 had a
354 negative impact on the maximum growth rate, decreasing the maximum growth rate by 37% (Figure 3).
355 Over time, the maximum growth rates of strains KP209 and KV402 decreased 13.4% and 9.5%,
356 respectively, compared to the maximum growth rate of the colistin-susceptible strain. In strain KP040, an
357 increase of 10.0% in maximum growth rate was observed during the course of the *in vitro* evolution
358 experiment.

359

360 **Colistin-resistant *K. pneumoniae* complex strains have lipid A that is modified through**
361 **hydroxylation, palmitoylation and addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N)**

362 To determine the modifications to lipid A in the colistin-resistant strains, we performed
363 MALDI-TOF analysis on lipid A isolated from the colistin-susceptible strain, and the axenic strain of the
364 last day of the *in vitro* evolution experiments. The MALDI-TOF spectra of lipid A isolated from
365 colistin-susceptible strains (Figure 4A), showed a dominant peak from hexa-acylated lipid A
366 (mass-to-charge ratio (m/z) 1824), corresponding to two glucosamines, two phosphates, four 3-OH-C₁₄ and
367 two C₁₄ acyl chains (50). Additional minor peaks in the MALDI-TOF spectrum of the susceptible strains
368 could be observed at m/z 1840, corresponding to the hydroxylation (m/z 16) of one of the C₁₄ acyl-groups

369 of hexa-acylated lipid A (m/z 1824), and at m/z 2063 (in KP209 and KP257), corresponding to a
370 hepta-acylated lipid A, with an additional acylation of lipid A (m/z 1824) with a palmitoyl group (m/z 239).

371 All the MALDI-TOF spectra of lipid A isolated from colistin-resistant strains show additional
372 peaks (Figure 4B), indicating the modification of their lipid A. In the spectra of colistin-resistant KP209
373 and KV402, lipid A m/z 1955 was observed, indicating addition of L-Ara4N (m/z 131) to the hexa-acylated
374 lipid A m/z 1824. In colistin-resistant KV402 lipid A m/z 1850 was observed, consistent with hexa-acylated
375 lipid A m/z 1824 with one C₁₆ acyl chain (Figure 4C). The peak at m/z 1866 in the MALDI-TOF spectra of
376 colistin-resistant KP040 and KP257 was consistent with hydroxylation of lipid A m/z 1850.

377

378 **Development of colistin resistance is associated with increased LL-37 resistance and virulence in a *C.***
379 ***elegans* survival model.**

380 To determine the impact of colistin resistance on virulence characteristics of the *K.*
381 *pneumoniae* complex strains, we first determined the susceptibility of the strains to the human cathelicidin
382 antimicrobial peptide LL-37. We observed that three of the four colistin-resistant strains (KP209, KP040,
383 and KP257) showed a decreased susceptibility to killing by LL-37 compared to their colistin-susceptible
384 parental strains (Figure 5). In contrast, development of colistin resistance in strain KV402 did not affect
385 susceptibility to LL-37.

386 To investigate the possible consequences of colistin resistance on virulence, we exposed the
387 nematode *C. elegans* strain CF512 to the colistin-susceptible/resistant strain pairs. *C. elegans* had a
388 decreased lifespan on a lawn of colistin-resistant KP209 (Figure 6) and KP040, compared to their
389 colistin-susceptible strains. Survival of *C. elegans* was not affected by growth on colistin-resistant strains
390 derived from KP257 and KV402, compared to the colistin-susceptible parental strains.

391 **Discussion**

392 Colistin plays a pivotal role in public health due to its last-resort status for treatment of infections
393 with multidrug-resistant Gram-negative bacteria. The increasing number of reports of *K. pneumoniae*
394 strains that have acquired resistance to multiple antibiotics, including colistin, is thus a cause for increasing
395 concern (7–9, 58). In this study, we aimed to study the potential to evolve colistin resistance in clinical *K.*
396 *pneumoniae* isolates. Due to the difficulties in generating targeted mutants in these multidrug-resistant
397 clinical isolates, we were limited to *in vitro* evolution experiments to identify mutations associated with
398 colistin resistance. We observed the swift development of colistin resistance through diverse evolutionary
399 trajectories. Development of colistin resistance had no, or only a minor, impact on maximum growth rate
400 in three out of four *in vitro* evolution experiments performed here. This suggests that colistin may rapidly
401 lose its effectiveness in the treatment of infections caused by multidrug-resistant *K. pneumoniae* complex
402 strains as fitness costs associated with colistin resistance seem limited.

403 We observe that mutations associated with an increase in MIC of colistin seem confined to genes
404 from functional groups involved in the synthesis and modification of LPS, and the synthesis of capsular
405 polysaccharides, which are both important surface-associated structures. In the genes encoding the PhoPQ
406 two-component regulatory system, which have a role in regulating modifications of LPS and contribute to
407 colistin resistance in Enterobacteriaceae (21, 59), we found variations in both PhoP (a D191N substitution),
408 and PhoQ (a G385S substitution, and a 12-bp deletion). The G385S PhoQ substitution has previously been
409 described in a colistin-resistant clinical *K. pneumoniae* strain (60). We also found that a novel integration
410 of an IS5 element in the promoter region associated with the genes encoding CrrAB and CrrC coincides
411 with increase in MIC of colistin. The IS5 element can influence the transcriptional activity of the genes
412 located near its integration site (61). The activity of PmrAB may be influenced by CrrAB through CrrC
413 (22, 62). In line with previous observations, in which insertions of IS elements were associated with

414 resistance to colistin, we hypothesize that the insertion of IS5 may lead to increased expression of CrrAB
415 and/or CrrC, and thus cause colistin resistance (28).

416 We observed that the inactivation of *wzc* of the K-locus, by the *IS102* element coincides with an
417 increase in the MIC of colistin. In *E. coli*, Wzc is involved in the synthesis and export of extracellular
418 polysaccharides containing colanic acid (63), but also the phosphorylation of other endogenous proteins
419 (64). Wzc has previously been hypothesised to be involved in colistin resistance in *E. coli*, and it may act
420 similarly in *K. pneumoniae* (64–66). The loss of Wzc may potentially cause colistin resistance through two
421 mechanisms. A reduction in the export of colanic acid units (the building blocks of *K. pneumoniae* capsule),
422 can lead to the accumulation of colanic acid metabolic intermediates, including UDP-glucuronic acid. This
423 accumulation has been hypothesised to lead to an increased flux towards biosynthesis of UDP-L-Ara4N,
424 resulting in the modification of lipid A with L-Ara4N (67). Alternatively, the absence or reduction of
425 negatively charged colanic acid residues on the cell surface could lower local concentrations of positively
426 charged colistin molecules, thereby reducing damage to the outer membrane (67). Further studies are
427 needed to fully characterize the interplay between the *Klebsiella* capsule and colistin resistance. In addition
428 to the inactivation of *wzc*, we observed a 12-bp insertion in the highly-conserved *rho* gene, encoding the
429 transcription termination factor Rho. Rho has not been previously linked to colistin resistance, but
430 mutations in *rho* may have pleiotropic effects on transcription (68), which could influence the expression
431 of genes involved in, or may compensate for fitness costs caused by colistin resistance.

432 Notably, we did not find any alterations in *mgrB*, which is an otherwise important mechanism
433 through which colistin resistance may occur in nosocomial *K. pneumoniae* complex strains (20, 25–27, 69).
434 Nevertheless colistin-resistant clinical *K. pneumoniae* isolates without mutations in *mgrB* are also
435 frequently encountered (60, 62, 70–73). We can only speculate on the reasons for the absence of *mgrB*
436 mutations in our *in vitro* evolution experiments. The relatively short duration of this experiment performed
437 with a limited number of strains, likely implies that we have not covered all potential colistin resistance

438 mechanisms in *K. pneumoniae*. Due to these limitations and the lack of replicate experiments, we cannot
439 make any conclusions on the repeatability or the need for a specific order in these mutational pathways.

440 The impact of developing colistin resistance through the observed mutations might extend past the
441 inability to treat the infection through antibiotic therapy, as we show in this study that modifications to
442 lipid A may reduce the susceptibility to antimicrobial peptides and increase virulence. However, the
443 mechanisms behind the differential effects on virulence of colistin resistance in the *K. pneumoniae* complex
444 are not fully understood and are deserving of further study. A single *K. variicola* isolate was included in
445 this study. While *K. variicola* can cause life-threatening infections in immunocompromised individuals (5),
446 it remains currently understudied. Additional studies into the mechanisms of colistin resistance and their
447 impact on fitness and virulence may be warranted in this species.

448 The emergence and spread of colistin resistance could complicate future treatments of infections
449 caused by multidrug-resistant Gram-negative bacteria. Our study indicates that in the *K. pneumoniae*
450 complex multiple evolutionary trajectories towards colistin resistance exist, without negatively impacting
451 fitness or virulence characteristics. Our data highlight the remarkable adaptive abilities of strains in the *K.*
452 *pneumoniae* complex, which makes them a nosocomial pathogen of considerable importance. Future
453 studies may lead to the development of novel therapeutics to specifically target colistin resistance
454 mechanisms, which may be essential to lengthen the clinical lifespan of colistin as a last-resort drug in
455 treatment of *K. pneumoniae* infections.

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469

470 **Competing interests**

471 The authors have declared that no competing interests exist.

472

473 **Author contributions**

474 A.B.J., D.J.D. and G.M. performed experiments and analysed data. A.B.J. and M.R.C.R performed
475 bioinformatic analyses. S.H.M.R., J.A.B., and W.v.S designed experiments. A.B.J., M.J.M.B, S.H.M.R.,

476 R.J.L.W., J.A.B. and W.v.S. wrote the manuscript. All authors reviewed and approved the manuscript
477 prior to submission.

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- 683

684

685 **Figure and table legends**

686 **Figure 1**

687 ***K. pneumoniae* complex strains: metadata, presence of antibiotic resistance genes, and**
688 **core-genome phylogenetic analysis.** A) Overview of the isolates used in this study, including the date
689 and source of isolation, MLST type, and the initial MIC determined. MLST typing of strain KV402
690 resulted in an incomplete MLST profile, so no conclusive ST could be assigned. NA, not applicable. B)
691 Antibiotic resistance genes detected in *K. pneumoniae* complex strains sequenced as part of this study.
692 Classes of antibiotic resistance genes are indicated as follow: BLA, beta-lactam resistance genes; QLN,
693 quinolone resistance genes; FOS, fosfomycin resistance genes. The strains did not carry acquired colistin
694 resistance genes of the *mcr*-family. C) Midpoint-rooted phylogenetic tree representing the 1.3-Mbp
695 core-genome alignment of 41 *K. pneumoniae* complex. Taxonomic phylogroups of the *K. pneumoniae*
696 complex (2) are indicated along the branches. The strains used in this study are highlighted in red.

697

698 **Figure 2**

699 **Population analysis of mutations during *in vitro* evolution in the presence of colistin.** For
700 each strain, and each day of the *in vitro* evolution experiment (performed in LB), the positions that have
701 mutated compared to the colistin-susceptible strain are indicated. For SNPs and indels, the number of
702 reads supporting a mutation at a given location was used to estimate the abundance of the mutation. Novel
703 integrations of IS elements are also indicated. For mutations not located in a coding sequence, nearby
704 coding sequences are indicated. Mutations and IS element integrations observed in the axenic, strain
705 isolated daily from each population are indicated by a blue border. The MIC of colistin (determined in
706 MHCAB) for each axenic strain isolated from the *in vitro* evolution population is indicated. The MIC

707 values represent the mode from three independent experiments performed in duplo. Hyp. protein:
708 hypothetical protein.

709

710 **Figure 3**

711 **Maximum growth rate of colistin resistant evolved strains.** Optical density at 600 nm (OD_{600})
712 was measured every 7.5 minutes during growth in LB. Representative data of three individual
713 experiments, performed in triplicate are shown. Mean and standard deviations are shown. A parametric
714 one-way ANOVA with Dunnett's multiple correction was used for the statistical analysis of the
715 differences in growth rates between the axenic strains isolated from each day of the *in vitro* evolution
716 experiment and the colistin-susceptible parental strain. Outcomes of the statistical analysis are indicated
717 by asterisks: $p < 0.05$ (*), < 0.01 (**), < 0.001 (***), or < 0.0001 (****).

718

719

720 **Figure 4**

721 **Lipid A modifications in colistin-susceptible and colistin-resistant strains.** MALDI-TOF
722 spectra showing the mass-to-charge (m/z) ratio values of the isolated lipid A from (A)
723 colistin-susceptible, and (B) colistin-resistant axenic strains, isolated from the cultures of the last day of
724 the *in vitro* evolution experiment. C) Proposed chemical structures of lipid A-moieties corresponding to
725 the observed m/z -values in the MALDI-TOF spectra. Modifications relative to the unmodified
726 hexa-acylated lipid A corresponding to m/z value 1824 are depicted in red. Hydroxylation of an acyl-
727 chain adds 16 to the m/z ratio, 4-amino-4-deoxy-L-arabinose adds 131, acylation with palmitate adds 239.

728

729 **Figure 5**

730 **Susceptibility of colistin-susceptible and colistin-resistant strains to the human cathelicidin**

731 **LL-37.** Strains were incubated for 90 minutes in 25% LB at 37°C with or without the addition of 50
732 µg/ml LL-37. Viability was assessed by determination of the number of colony-forming units. The
733 non-parametric Mann-Whitney test was used as statistical test and significance was defined as a p-value
734 of < 0.05 (*), < 0.01 (**), <0.001 (***), or <0.0001 (****).

735

736 **Figure 6**

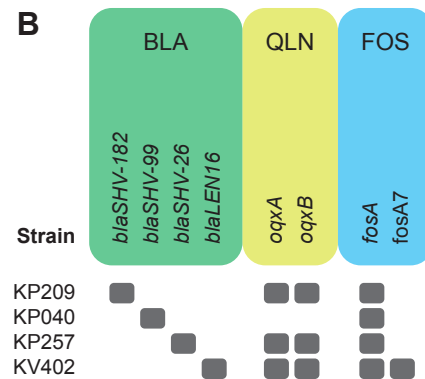
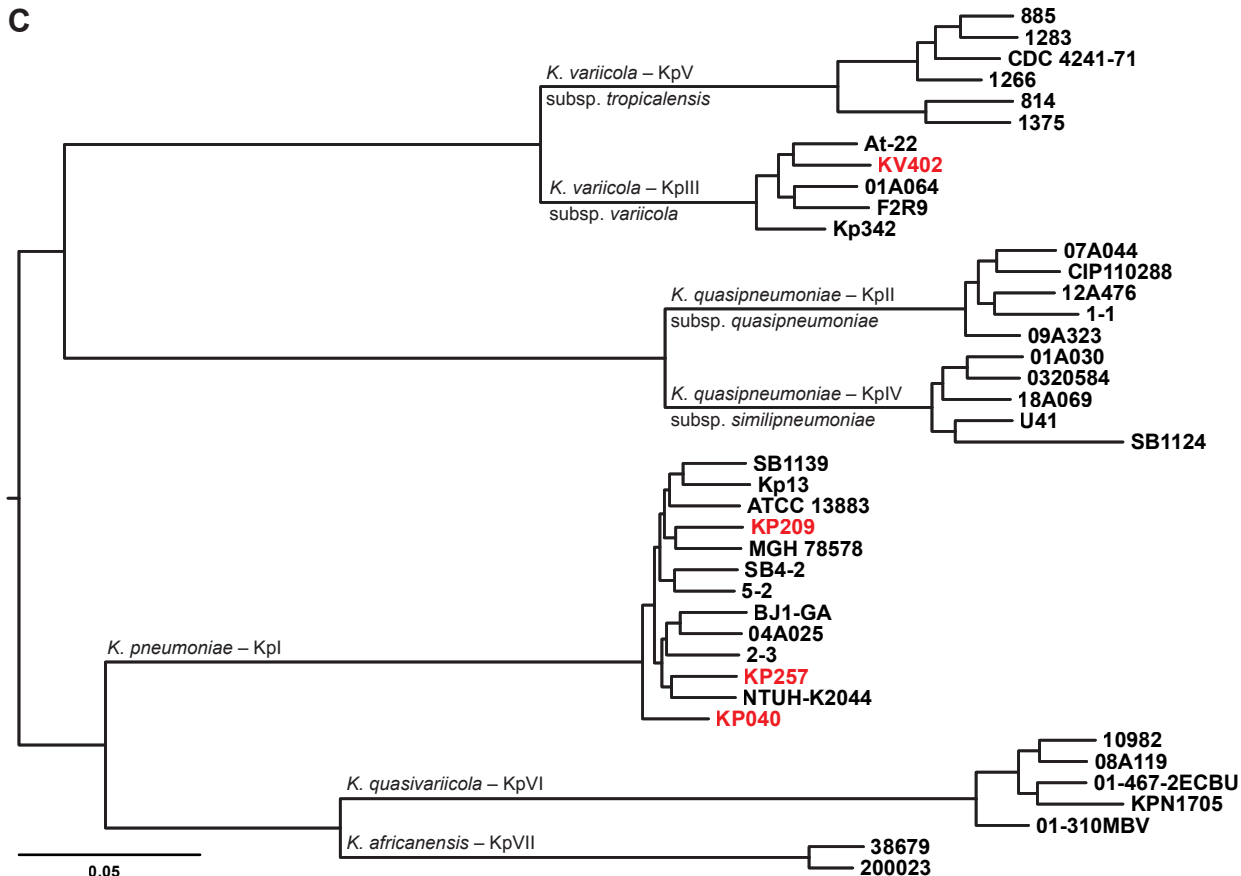
737 **Survival of *C. elegans* on lawns of *K. pneumoniae* colistin-susceptible and colistin-resistant**

738 **strains complex strains.** *C. elegans* CF512 were kept on a lawn of colistin-susceptible (green) and
739 colistin-resistant (red) *K. pneumoniae* complex strains. Survival was scored over a period of 15 days. The
740 data represent three independent experiments in which a total of 129 (in colistin-susceptible KP209), 118
741 (colistin-resistant KP209), 106 (colistin-susceptible KP040), 127 (colistin-resistant KP040), 127
742 (colistin-susceptible KP257), 131 (colistin-resistant KP257), 100 (colistin-susceptible KP402), and 102
743 (colistin-resistant KP402) *C. elegans* nematodes were used. Statistical significance according to
744 Mantel-Cox log-rank test is indicated. Statistical significance was defined as a p-value < 0.05.

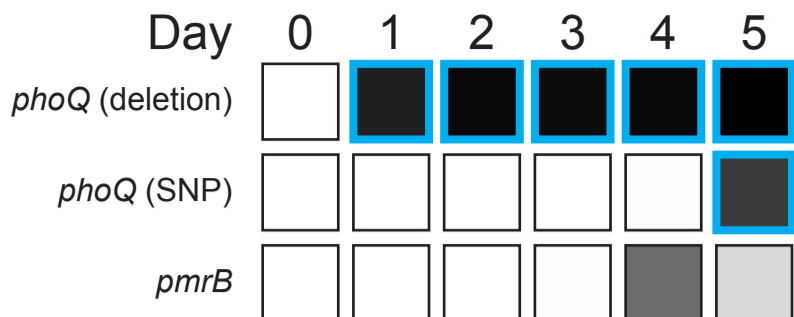
745

A

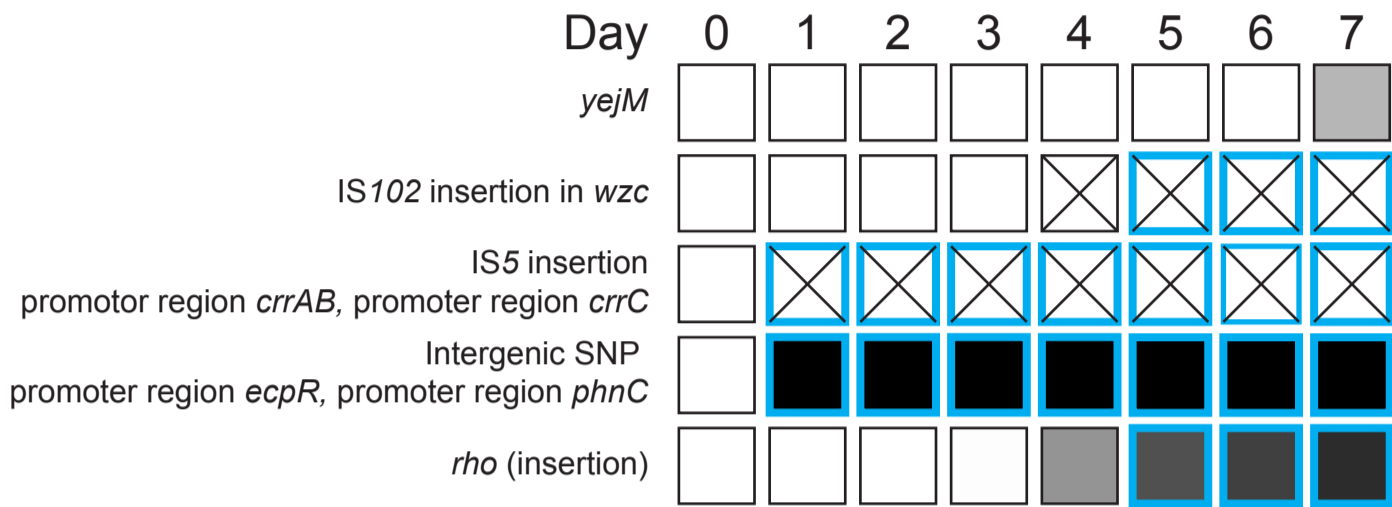
Strain	Date of isolation	Isolation source	MLST type	MIC of colistin (mg/L)	Reference
KP209	17-09-2013	Urine	11	0.5	This study
KP040	09-09-2013	Faeces	10	1	This study
KP257	27-09-2013	Pus	3030	1	This study
KV402	19-09-2013	Urine	NA	0.5	This study

B**C**

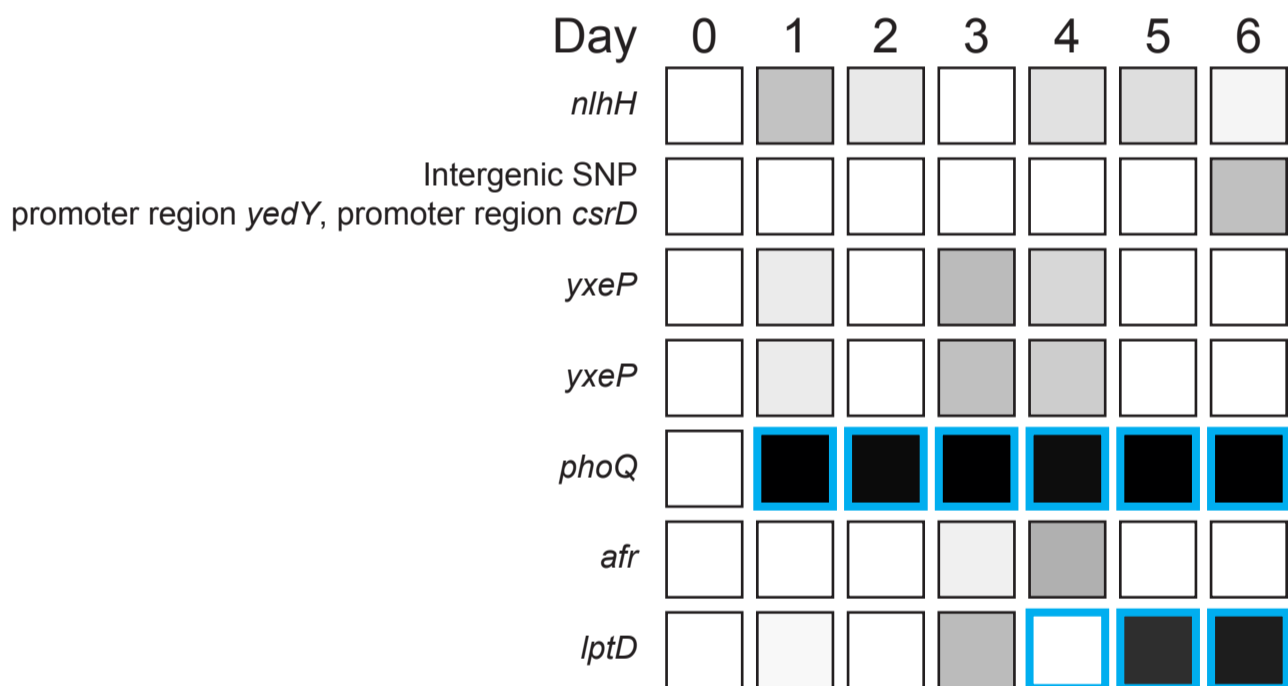
KP209



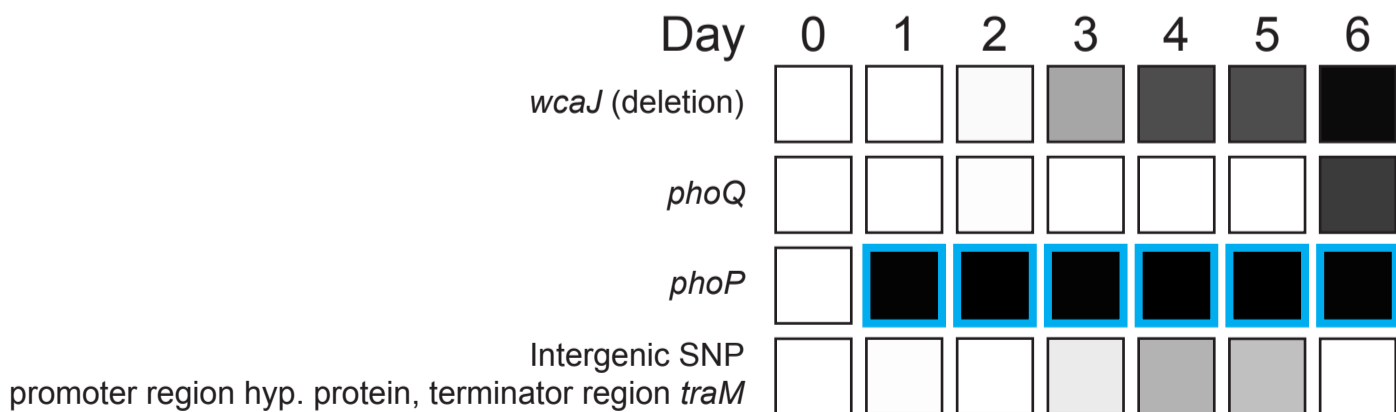
KP040



KP257



KV402



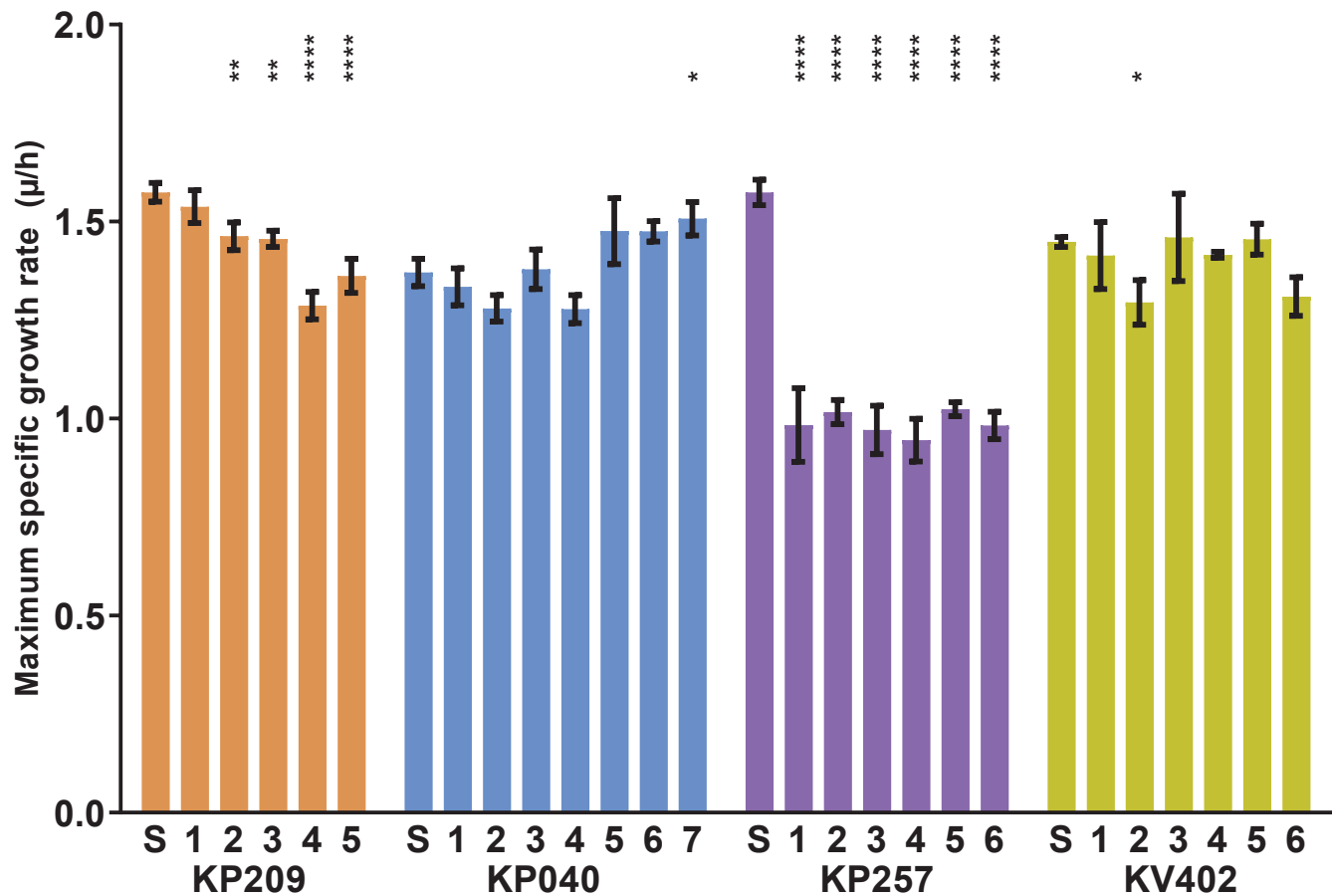
0% Reads supporting mutation 100%



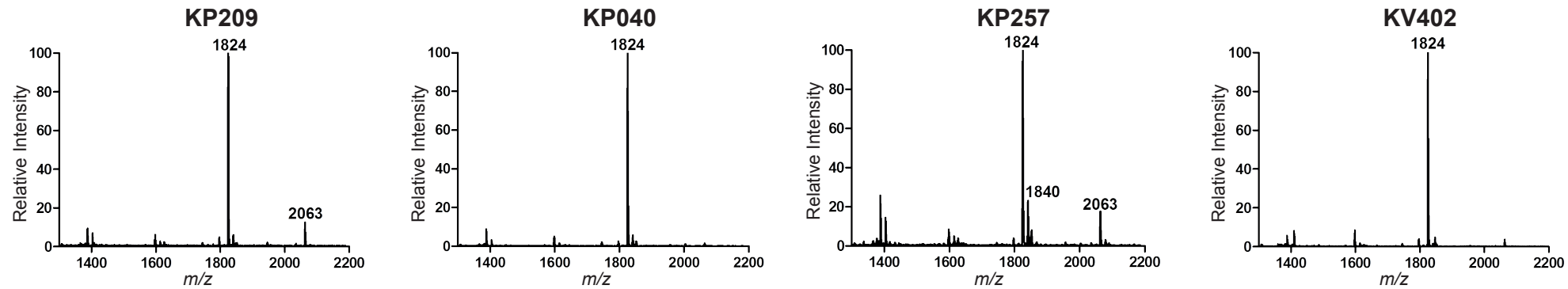
Insertion of IS element



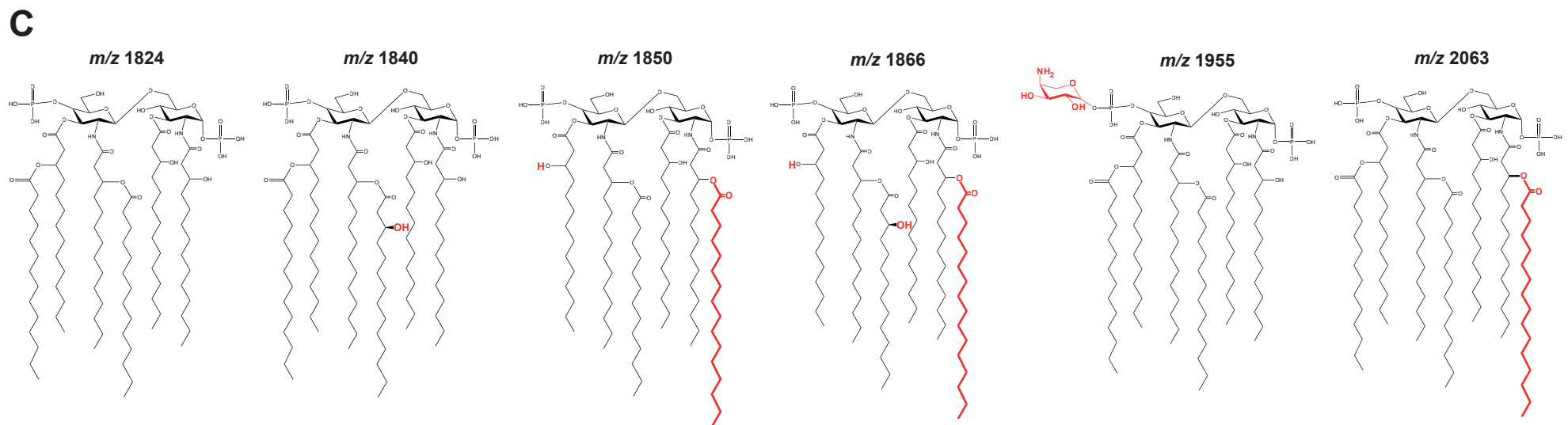
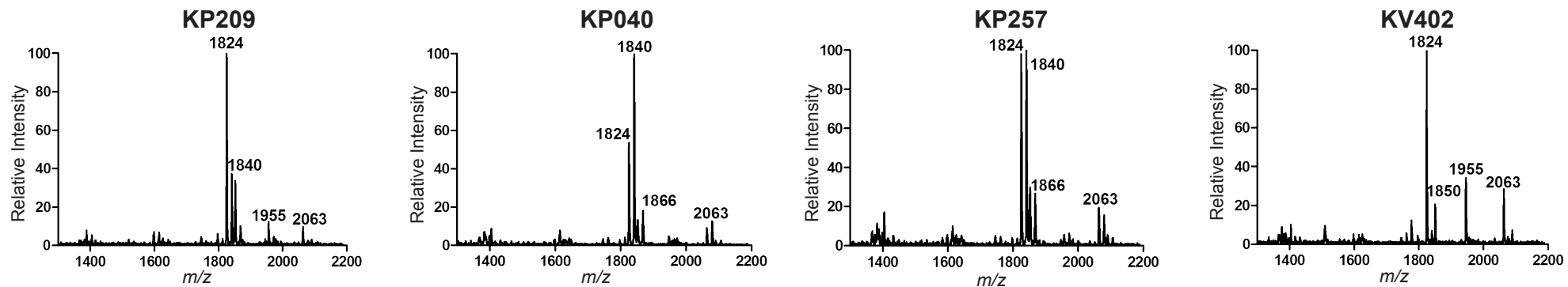
Mutation observed in axenic strain isolated from this population

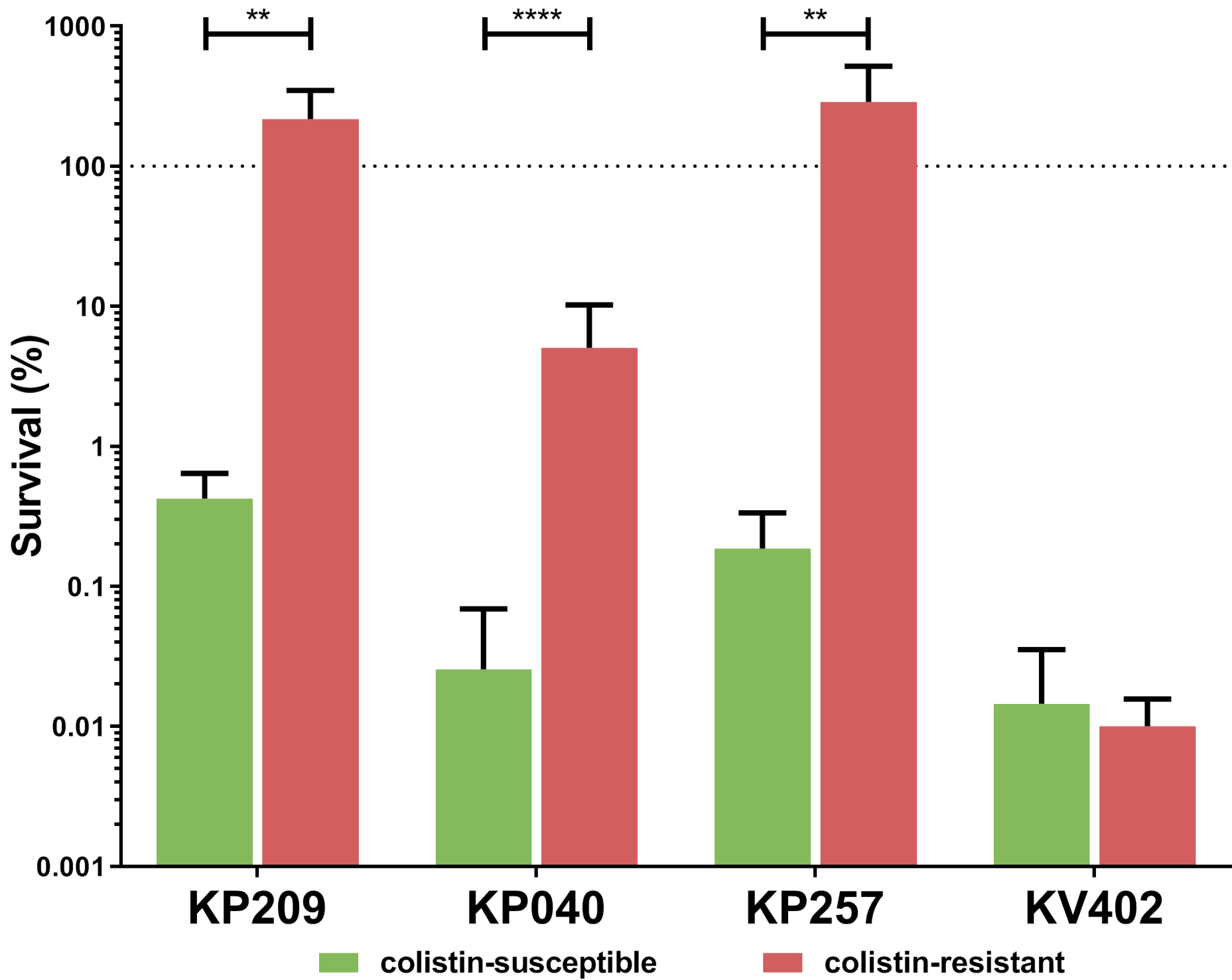


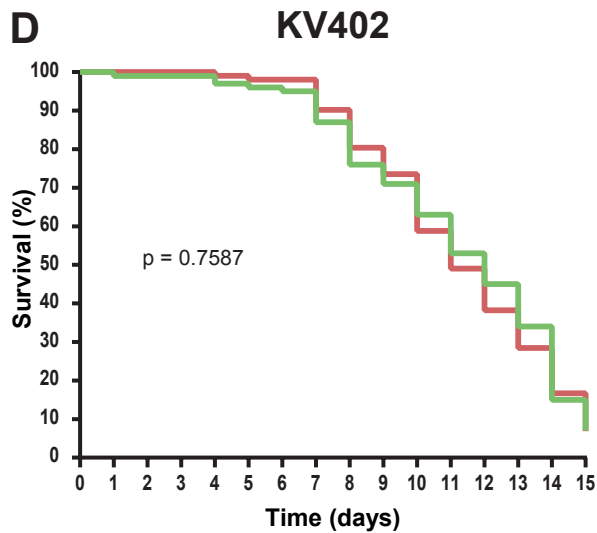
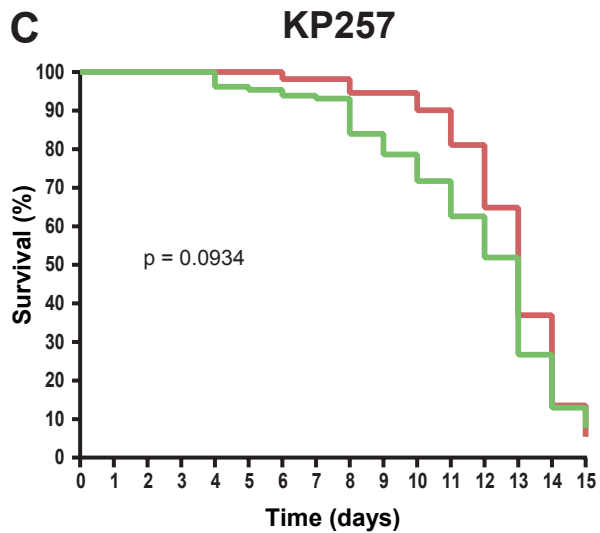
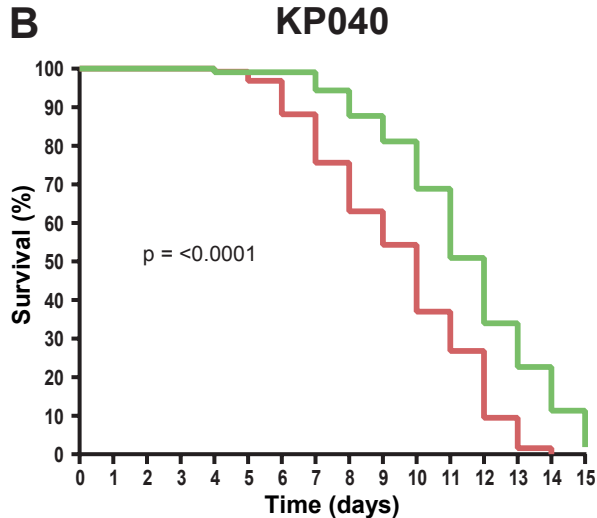
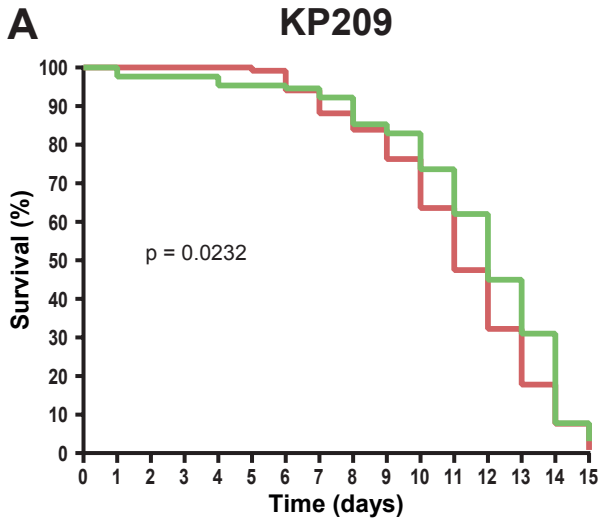
A colistin-susceptible



B colistin-resistant







— Colistin-susceptible

— Colistin-resistant