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1 ***ramR* deletion in an *Enterobacter hormaechei* isolate as a consequence of therapeutic failure**
2 **to key antibiotics in a long-term hospitalized patient**

3
4 Running title: *ramR* alteration in a multi-drug resistant ECC isolate

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21
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28

29 **ABSTRACT**

30 Genome changes are central to the adaptation of bacteria especially under antibiotic pressure.
31 The aim of this study was to report phenotypic and genomic adaptation undergone by
32 *Enterobacter hormaechei* clinical strain that became highly resistant to key antimicrobials during
33 a four months period in a patient hospitalized in intensive care unit (ICU). All six clinical *E.*
34 *hormaechei* strains isolated in one ICU-hospitalized patient have been studied. MICs regarding
35 17 antimicrobial molecules have been measured. Single nucleotide polymorphism (SNP), was
36 determined on the sequenced genomes. The expression of genes involved in antibiotic resistance
37 among *Enterobacter cloacae* complex strains were determined by qRT-PCR. All the strains
38 belonging to the same Sequence-Type 66 and were distant by a maximum of nine SNP. After
39 three months of hospitalization, three strains presented a significant increase in MICs regarding
40 ceftazidime, cefepime, temocillin, ertapenem, tigecycline, ciprofloxacin and chloramphenicol.
41 Those resistant strains did not acquire additional antibiotics resistant genes but harbored a 16 bp
42 deletion in the *ramR* gene. This deletion led to the up expression of RamA, AcrA, AcrB, TolC
43 and the down expression of OmpF. The $\Delta ramR$ mutant harbored the same phenotype as the
44 resistant clinical strains regarding tigecycline, chloramphenicol and ciprofloxacin. The increased
45 expression of RamA due to partial deletion in the *ramR* gene led to a cross resistance phenotype
46 by an increase of antibiotics efflux through the AcrAB-TolC pump and the decrease of
47 antibiotics permeability by porin OmpF. *ramR* appears to be an important adaptative trait for *E.*
48 *hormachei* strains.
49

50 **INTRODUCTION:**

51 Members of the *Enterobacter cloacae* complex (ECC) are major opportunistic pathogens,
52 especially in patients hospitalized in intensive care units (ICUs). Within this complex, three
53 clusters (C3, C6 and C8) that all correspond to *Enterobacter hormaechei* species are mainly
54 recovered from clinical samples and are responsible for infections and hospital outbreaks (1).
55 Furthermore, many ECC strains have become resistant to broad-spectrum cephalosporins by
56 overproduction of chromosomal cephalosporinase (AmpC) and/or by acquisition of genes coding
57 for extended-spectrum β -lactamases (ESBL). Even more alarming is the emergence of
58 carbapenemase-producing *Enterobacteriaceae* (CPE), which has increased the focus on ECC
59 strains. In addition, multidrug efflux pumps (especially those belonging to the Resistance-
60 Nodulation-Cell-Division [RND] family) and membrane decreased permeability (due to
61 modifications in porin concentration) play a major role in the resistance of Gram-negative
62 bacteria to a wide range of antibiotics (2–6). Moreover, genes involved in the regulation of efflux
63 pumps and porin expression can also contribute to the adaptation of strains to their environment
64 and to avoid the host immune system (7, 8). Among these regulator genes, *ramAR* locus
65 belonged to the AraC/XylS-transcriptional activator family (9). RamA adjusts the membrane
66 permeability by modulating the concentrations of the efflux pump AcrAB-TolC as well as the
67 porin OmpF (10). The expression of RamA is negatively controlled by RamR. Among several
68 *Enterobacteriaceae* species it has been described that mutations in *ramR* drive to an over
69 expression of RamA which leads to a cross-resistance phenotype with an increase of MICs
70 regarding β lactams, fluoroquinolones, tigecycline, and chloramphenicol (11–13). Here, we
71 present how a *E. hormaechei* strain evolved rapidly *in vivo* under multiple antibiotics treatment
72 administered in one patient.

73 In January 2018, a 75-year-old man was admitted to a surgical ICU after vascular surgery. The
74 patient quickly developed several septic complications, such as bacteraemia, nosocomial
75 pneumonia and peritonitis caused alternatively by *E. hormaechei* and methicillin-resistant
76 *Staphylococcus epidermidis* strains, for which he received an escalation of twelve different
77 antimicrobial agents (piperacillin/tazobactam, imipenem, meropenem, ceftazidime/avibactam,
78 amikacin, cotrimoxazole, vancomycin, linezolid, daptomycin, erythromycin, ceftaroline and
79 levofloxacin) until he died of septic shock four months after his admission. During his
80 hospitalization, six *E. hormaechei* strains were isolated from stools, blood culture, urines,
81 bronchoalveolar and peritoneal fluids. Initially, the strains exhibited an ESBL phenotype and
82 then became resistant to almost all antibiotics tested. The aim of this study was to decipher the
83 phenotypic and genomic evolution of these strains, which acquired a multidrug resistance
84 (MDR) phenotype *in vivo*.

85

86 **RESULTS:**

87 **Antibiotics and antiseptics susceptibilities:**

88 Comparison of antibiotics susceptibility profiles showed two distinct groups: strains FY01 to
89 FY03 (group I) were susceptible to chloramphenicol (CHL) and tigecycline (TIG) (MICs of 4
90 and 0.25 mg/L, respectively), whereas FY06 to FY10 (group II) were resistant (MICs of 32 and 2
91 mg/L, respectively) (Table 1). In addition, significant differences in MICs between the two
92 groups of isolates were observed for ciprofloxacin (CIP) (MICs increased from 2 to 32 mg/L
93 (16-fold)) (Table 1). Lower changes were noted for some β lactams, such as temocillin (TEM) (4-
94 fold), PTZ (2-fold), cefepime (FEP) (2-fold) and ertapenem (ETP) (2-fold) (Table 1). Regarding
95 antiseptic testing, reduced susceptibility to CHX (MIC 16 mg/L) was observed for all the clinical

96 strains in comparison to the reference strain (ATCC13047, MIC 2 mg/L). Group II strains
97 showed a two-fold increase in MIC to CHX (32 mg/L) compared to group I strains (Table 1).

98

99 ***In silico* typing:**

100 The six strains were identified *in silico* as *Enterobacter hormaechei* subsp. *xiangfangensis*; they
101 all belonged to Sequence Type (ST) 66 and the C6 Hoffman cluster. WGS analyses confirmed
102 that these six strains (isolated from a unique clinical specimen from the same patient) were
103 isogenic with a maximum of nine SNPs distance between all the strains, and only one SNP was
104 observed between the two groups.

105

106 **Antibiotics resistance genetic determinants:**

107 The six strains shared the same resistome: *bla*_{CTX-M15}, *bla*_{TEM-1B}, *bla*_{OXA-1}, *aac(3)-IIa*, *aac(6')-Ib-*
108 *cr*, *aph(3'')-Ib*, *aph(6)-Id*, *sul2*, *dfrA14* and *qnrB1*. Regarding known point mutations targets, a
109 unique nonsynonymous mutation in GyrB (Phe746Ser) was found among the strains of the group
110 II. This mutation was outside the quinolone resistance-determining region (QRDR) of *gyrB* and
111 could not explain the CIP MIC modifications. Moreover, no mutations or insertions/deletions
112 were observed in genes involved in antibiotic resistance (Supplementary Table 2).

113 However, by comparing the whole genomes of the group I to those of the group II, a 16-bp
114 deletion in the LI63_RS07965 gene coding for a TetR-like transcriptional regulator was observed
115 in the group II. This gene is a homologue of the *ramR* gene of *Klebsiella pneumoniae* Ecl8 (59%
116 identical) and *Salmonella enterica* subsp. *enterica* serovar Kentucky CDC 19 (63% identical)
117 and is called *ramR* in this study. This deletion was responsible for a shift in the nucleotide
118 sequence reading frame leading to a loss of the first 69 N-terminal amino-acids (aa) of RamR.

119 Immediately downstream from *ramR* gene, there is a 1,104-bp gene, *romA*, which encodes a 367-
120 aa metallo-hydrolase. This gene is similar to the *romA* genes of *K. pneumoniae* (70% identical)
121 and *Citrobacter freundii* (21% identical). Downstream *romA*, there is *ramA* a 342-bp gene which
122 encodes a 113-aa protein RamA that belonged to the AraC/XylS-transcriptional activator family.

123

124 **Transcriptional consequences of *ramR* deletions:**

125 The qRT-PCR experiments evaluated the regulatory consequences of *ramR* alteration by
126 comparing gene expression between group I and group II strains. These experiments highlighted
127 that the loss of the N-terminal portion of RamR among the strains FY06 to FY10 led to the
128 overexpression of *ramR* itself (fold change (FC): 26), *romA* (FC: 680), *ramA* (FC: 453), *micF*
129 (FC: 124), *acrA* (FC: 9), *acrB* (FC: 7), *tolC* (FC: 4) and a reduced expression of *ompF* (FC: 0.08)
130 (Figure 2). No difference in genes expression have been observed regarding chromosomal *ampC*,
131 *ompA*, *ompC*, as well as another AraC/XylS-transcriptional activator *marA* (Figure 2). These
132 results suggested that dysregulation in *ramRA* locus could explain the emergence of a cross-
133 resistance phenotype by modifying membrane permeability by increasing efflux pump
134 expression and decreasing OmpF concentration.

135

136 ***Enterobacter cloacae* ATCC 13047 mutant analyzes:**

137 To decipher the resistance mechanism in the *E. hormaechei* strains, one mutant of *E. cloacae*
138 ATCC 13047 deleted for the gene ECL_RS15545 was constructed. Thus, MICs of CHL, TIG
139 and CIP were determined for the $\Delta ramR$ (ATCC13047 Δ ECL_RS15545) strain. *E. cloacae*
140 ATCC 13047 cells in which *ramR* was deleted became significantly more resistant (FC \geq 4) to
141 CHL, TIG and CIP like the clinical strains (FY06 to FY10) harboring a 16 pb deletion in *ramR*

142 (Table 1). Furthermore, the transformation of the ATCC13047 Δ ECL_RS15545 with the
143 pBAD202 plasmid containing the *ramR* sequence of FY08 did not allow any restoration of the
144 MICs (Table 1). In order to further analyze the role of the RamR regulator, two clinical isolates
145 (FY01 and FY10) were transformed with the pBAD202 vector containing *ramR* cloned with its
146 promoter region (Table 1). The MICs of CHL, TIG and CIP for FY10 complemented strains
147 decreased reaching the same values as the FY01 strain (Table 1). Finally, in the presence of Phe-
148 Arg- β -naphthylamide (PA β N), there was a significant decrease in the MICs of CHL (FC: 8), TIG
149 (FC: 8) and CIP (FC: 4) for the ATCC13047 Δ ECL_RS15545 and FY10 strains, suggesting the
150 involvement of RND efflux pumps such as AcrAB-TolC (Table 1).

151 These results allowed us to hypothesize that the prescription of antibiotics during February and
152 March 2018, such as β lactams and levofloxacin (figure 1), led to the selection of clinical strains
153 with the *ramR* mutation and an over expression of the RamA controlled cascade, i.e the
154 expression of different genes involved in efflux pump and decrease porin expression (Figure 2).

155

156 **DISCUSSION:**

157 This article provides a description of an *in vivo* evolution of a unique *E. hormaechei* clinical
158 strain under an important antibiotic pressure by the partial deletion of the 3' end of *ramR* gene
159 leading to a cross-resistance phenotype. Dysregulation among the *ramRA* locus and the
160 consequences have already been described among several species such as *K. pneumoniae*, *S.*
161 *enterica*, *C. freundii*, *K. aerogenes* and ECC (9, 11, 12, 14). RamA belongs to the AraC-XylS
162 transcriptional activator family, and, like MarA, controls the membrane permeability by both:
163 regulating the OmpF porin expression via the antisense RNA *micF* and the efflux pump AcrAB-
164 TolC (10). RamA could be over expressed by two main mechanisms; first deletions in a region

165 located upstream his gene, near by the marbox could alter the RamA self-regulation (11) but
166 there was no alteration in this region among the clinical strains (data not show). Secondly, RamA
167 could be over-expressed by the non-functionality of RamR (12, 14). RamR is composed by two
168 functional regions: the N-terminal one corresponding to the helix-turn-helix (HTH) domain
169 which is the DNA binding site whereas the second one is required to the substrate recognition
170 and binding (11, 15). In our study, RamR lost its first 69 N-Terminal amino-acids. As a
171 consequence, RamR could not bind the DNA and could not inhibit RamA expression. To the best
172 of our knowledge, *ramR* mutations conferring antibiotic resistance has not been described among
173 *E. hormaechei* strains yet. However, these events have already been described among *K.*
174 *pneumoniae*, *S. enterica* serovar Typhimurium and *K. aerogenes* (11–13) with a variety of
175 mutations, which could allow bacteria to quickly adapt to antibiotics pressure (11). The *E.*
176 *hormaechei* multi-resistant clinical strains may have been selected by β lactam and
177 fluoroquinolones treatments which were given during the February and March (Figure 1).
178 Results of the qRT-PCR confirmed that, a non-functional RamR lead to over-expression RamA,
179 AcrA, AcrB, TolC, and a reduce expression of OmpF as it has been previously described (15).
180 However, our results suggest that RamA over expression did not lead to a MarA increase
181 expression contrarily to what has been published in *K. aerogenes* (10). Moreover, it seems the N-
182 terminal deletion of RamR leads to its own over expression which has been inconstantly
183 described among *K. aerogenes* clinical and mutant strains (11). Taken together these results
184 suggest that RamR could contribute to the regulation of its own expression.

185 The role played by the pump AcrAB-TolC in the increase of the MICs of CHL, TIG and CIP
186 have been demonstrated by using the Pa β N (Table 1). Concerning the OmpF downregulation,
187 MICs of the β -lactam in the ATCC13047 Δ ECL_RS15545 strain did not change as it was among

188 the clinical strains. Indeed, the increase in the MICs of β lactam is only significant if the reduce
189 expression of OmpF is associated with another mechanism, such as the acquisition of an ESBL
190 enzyme and/or *ampC* derepression which was not the case in this mutant (16). Nevertheless, the
191 *ramR* complemented clinical strain FY10 recovered initial MICs including β lactams (Table 1).
192 Usually, bacterial adaptation is associated with horizontal genes transfers between bacteria in the
193 same microbiota. This work highlighted how a chromosomal mutation in *ramR* could be a
194 response to antibiotics selection pressure. Moreover, it has been described in *K. pneumoniae* that
195 RamA over expression allows the bacteria to hide to mouse immune system. Modifications in
196 *ramR* could provide multiple advantages to bacteria and need to be more analyzed among
197 clinical *Enterobacteriaceae* as it appears to be an important adaptative trait.

198

199 **CONCLUSION:**

200 Host adaptation through gene acquisition, genome degradation (gene inactivation or deletion) or
201 both is central to pathogen evolution. In the present study, we highlighted that (i) the
202 transcriptional regulator RamR of *E. hormaechei* is a repressor of the expression of *ramA* and (ii)
203 the *ramRA* locus appears to be the most potent regulator of cross-resistance phenotype for *E.*
204 *hormaechei*.

205

206 **METHODS**

207 ***Bacterial strains***

208 During a four-month period, six *E. hormaechei* strains were recovered from the same patient:
209 one from rectal swab and five from clinical samples. Details about the chronology of strains
210 isolations and the antibiotics prescriptions are available in Figure 1. Identification at the species

211 level was performed by using the MALDI-TOF mass spectrometry method (Bruker, Germany).
212 The reference strain *E. cloacae* subsp. *cloacae* ATCC 13047 (nucleotide accession number
213 NC_014121.1) was used to create mutants (17). The bacterial strains used in this study are listed
214 in Table 2.

215

216 ***Antimicrobial agents susceptibility test***

217 Antimicrobial susceptibility was examined using the microdilution method according to the
218 guidelines recommended by the European Committee on Antimicrobial Susceptibility
219 (EUCAST, <http://www.eucast.org/>). The antimicrobial agents or combinations tested included
220 piperacillin-tazobactam (PTZ), temocillin (TEM), ceftazidime (CAZ), cefotaxime (CTX),
221 cefepime (FEP), aztreonam (AZT), ertapenem (ETP), meropenem (MER), imipenem (IMP),
222 amikacin (AMK), gentamicin (GEN), ciprofloxacin (CIP), chloramphenicol (CHL) tigecycline
223 (TIG), colistin (CS), cetyl trimethyl ammonium chloride (CTAB) and chlorhexidine (CHX). All
224 tests were performed in triplicate. The MICs of CHL, TIG and CIP were also determined by the
225 broth microdilution method in the presence and absence of Phe-Arg- β -naphthylamide (PA β N) at
226 a concentration of 40 mg/L.

227

228 ***DNA sequencing and bioinformatics***

229 High-throughput whole-genome sequencing (WGS) was carried out on all *E. hormaechei* strains
230 at the “Plateforme de Microbiologie Mutualisée” (P2M) from the Pasteur International
231 Bioresources network (PIBNet, Institut Pasteur, Paris, France). DNA extraction was performed
232 using the MagNAPure 96 system (Roche, Germany). Libraries were prepared using the Nextera
233 XT kit (Illumina, USA), and sequencing was performed with the NextSeq 500 system (Illumina,

234 USA), generating 150 bp paired end reads. Alien Trimmer was used for reads trimming and
235 clipping (18). The quality of the filtered fastq files was evaluated using fastqc software
236 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Finally, genomes were *de novo*
237 assembled using SPAdes v 3.12 software using the recommended kmer size for Illumina 150 bp
238 paired reads (-k 21,33,55,77) (19). The quality of the genome assembly was assessed using Quast
239 software (20). Species of the sequenced bacteria were checked using the NCBI prokaryotic
240 genome annotation pipeline (21). Sequence type (ST) was determined using the multi-locus
241 sequence typing scheme (MLST) based on seven housekeeping genes: *dnaA* (chromosomal
242 replication initiator protein DnaA), *fusA* (elongation factor G), *gyrB* (DNA gyrase subunit beta),
243 *leuS* (leucine--tRNA ligase), *pyrG* (CTP synthase), *rplB* (50S ribosomal protein L2) and *rpoB*
244 (DNA-directed RNA polymerase subunit beta) (22). The cluster appurtenance, based on the
245 partial sequence of the *hsp60* gene, was determined using the blastn algorithm with the following
246 parameters: identity, 97%; coverage, 100% (23, 24). Acquired antibiotic-resistance genes and
247 point mutations were searched in the Center for the Genomic Epidemiology (CGE) using
248 Resfinder and PointFinder databases respectively (25). The genes described as involved in
249 antibiotic resistance among ECC strains are summarized in supplementary text one. All of them
250 were screened for using blastn with the following parameters: identity, 80%; coverage, 80%.

251 For the reads mapping process, the reference genome used was *E. hormaechei* subsp. *oharae*
252 strain 34978 (nucleotide accession number CP012165.1). The reads were aligned using Burrows-
253 Wheeler Aligner (26) version 0.7.17, and variant calling was performed using mpileup (27).

254

255 ***Construction of the knockout mutants and a multicopy plasmid library containing putative***
256 ***regulator ORFs***

257 Deletion of the putative TetR regulator (ECL_RS15545) was performed using the method
258 previously described which employs the Red helper plasmid pKOBEG (28–30). The primers
259 used in the study are reported in Supplementary Table 1. The TetR regulator (ECL_RS15545),
260 including its own promoter, was amplified by PCR using the primers listed in Supplementary
261 Table 1. The amplicon was then TA-cloned into the overexpression plasmid pBAD202 (low-
262 copy number plasmid: ~20 copies/cell) Directional TOPO (Invitrogen, France). *Escherichia coli*
263 TOP-10 cells (Invitrogen, France) carrying pBAD202 recombinants containing correctly oriented
264 inserts were selected on LB plates with 40 mg/L kanamycin. After purification, a plasmid
265 carrying the *ramR* regulator gene with its own promoter was used to transform the ECC
266 ATCC13047 strain and clinical isolates (Tables 1 - 2).

267

268 ***Gene expression by RNA quantification***

269 Total RNA was extracted from bacterial cells grown to the late exponential phase by using the
270 Direct-Zol RNA mini-prep kit (Zymo Research, USA). Residual chromosomal DNA was
271 removed by treating samples with the TURBO DNA-free kit (Life Technologies, France).
272 Samples were quantified using the NanoDrop One spectrophotometer (Thermo Fisher Scientific,
273 France). cDNA was synthesized from total RNA (~1 µg) using the QuantiTect reverse
274 transcription kit (Qiagen, Netherland) according to the manufacturer's instructions. Transcript
275 levels of the following genes *ramR*, *romA*, *ramA*, *micF*, *acrA*, *acrB*, *tolC*, *marA*, *marR*, *ompA*,
276 *ompC*, *ompF*, *ompR*, *ampC*, *bla_{CTX-M15}*, and *aac(6')-Ib-cr* were determined using primers
277 described in Supplementary Table 1. Note that the DNA binding sites of the primers used for
278 *ramR* were not affected by the 16 bp deletion. Fold changes were calculated using the delta-delta
279 Cycle threshold (CT) method using *rpoB* as housekeeping gene. Experiments were performed at

280 least three times. Differences of gene expression between strains FY06 to FY10 (group II) and
281 FY01 to FY03 (group I) were evaluated using Student's t-test on GraphPad Prism version 8. *P*-
282 values lower than 0.05 were considered statistically significant.

283

284 ***Ethics:***

285 This study has been conducted in compliance with the Helsinki Declaration (Ethical Principles
286 for Medical Research Involving Human Subjects) and was conducted in accordance with the
287 guidelines of research board of our teaching hospital. The patient's data were anonymously
288 reported. Because specimens used in this study were part of the routine patient management
289 without any additional sampling, and because patient's family provided no objection for his
290 samples to be used, this study did not need to be examined by an ethical committee and patients'
291 informed consent was not required.

292

293 ***Data availability:***

294 Genomes of this study and information about the strains are described in the following
295 bioproject: PRJNA623478 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA623478>). Genomes
296 used for *ramR*, *romA*, and *ramA* comparison were *Enterobacter hormaechei* subsp. *oharae* strain
297 34978 (NZ_CP012165.1, https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP012165.1), *Klebsiella*
298 *pneumoniae* subsp. *pneumoniae* strain Ecl8 (HF536482.1,
299 <https://www.ncbi.nlm.nih.gov/nuccore/HF536482.1>), *Salmonella enterica* subsp. *enterica*
300 serovar Kentucky str. CDC 191 (ABEI01000007.1,
301 <https://www.ncbi.nlm.nih.gov/nuccore/ABEI01000007.1>), *Citrobacter freundii* strain
302 FDAARGOS_61 (CP026045.1, <https://www.ncbi.nlm.nih.gov/nuccore/CP026045.1>).

303 Nucleotides sequences of genes described as involved in antibiotic resistance in ECC strains are
304 available in supplementary text one. The bioinformatics analyses did not depend on home-made
305 algorithms but on publicly available software which have been described above.

306

307 **CONFLICT OF INTERESTS**

308 The authors have no conflicts of interest to declare

309

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319

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TABLES

Table 1. Minimal Inhibitory Concentrations (MICs) of the studied strains:

	MICs (mg/L)																
	PTZ	TEM	CAZ	CTX	FEP	AZT	ETP	MER	IPM	AMK	GEN	CIP	CHL	TIG	CS	CHX	CTAB
<i>E. cloacae</i> ATCC13047	8	4	1	1	0.06	0.5	0.25	0.125	0.25	1	0.5	0.016	4	0.25	>256	2	32
13047ΔECL_RS15545 (<i>ramR</i>)	16	8	1	2	0.125	1	0.5	0.125	0.5	1	0.5	0.06(4)	16(4)	2(8)	>256	4	32
13047_pBAD202	8	4	1	1	0.06	1	0.25	0.125	0.25	1	0.5	0.016	4	0.25	>256	2	32
13047_pBAD202ΔECL_RS15545	8	4	1	1	0.06	1	0.25	0.125	0.25	1	0.5	0.016	2	0.25	>256	2	32
13047ΔECL_RS15545_pBAD202	16	8	1	2	0.125	1	0.5	0.125	0.5	1	0.5	0.06(4)	16(4)	2(8)	>256	4	32
13047ΔECL_RS15545_pBAD202 ΩFY08_Δ <i>ramR</i> ^c	16	8	1	2	0.125	1	0.5	0.125	0.5	1	0.5	0.06(4)	16(4)	2(8)	>256	4	32
FY01	64	4	32	256	16	32	0.25	0.125	0.5	2	32	2	8	0.5	0.125	16	32
FY02	64	8	32	256	32	64	0.25	0.125	0.5	2	32	2	4	0.25	0.125	16	32
FY03	64	4	64	128	32	64	0.25	0.125	0.5	2	32	2	4	0.25	0.25	16	32
FY06 ^b	64	16(4)	128	256	64(4)	64	0.5	0.125	0.5	2	32	32(16)	32(8)	2(8)	0.125	32	32
FY08 ^b	64	16(4)	64	256	64(4)	64	0.5	0.125	0.5	4	64	32(16)	32(8)	2(8)	0.125	32	32
FY10 ^b	128	16(4)	64	256	64(4)	64	0.5	0.125	0.5	4	64	32(16)	32(8)	2(8)	0.125	32	32
FY01_pBAD202	32	4	32	128	16	64	0.25	0.125	0.5	2	32	2	4	0.5	0.125	16	32
FY01_pBAD202ΔECL_RS15545	32	4	16	128	16	32	0.25	0.25	0.5	2	32	2	4	0.5	0.125	16	32
FY10_pBAD202	128	16	64	256	64	64	0.5	0.125	1	2	64	32	16	2	0.125	32	32
FY10_pBAD202ΔECL_RS15545	32	4(-4)	32	128	16(-4)	32	0.25	0.125	0.5	2	32	2(-16)	4(-4)	0.5(-4)	0.125	16	32
<i>E. cloacae</i> ATCC13047 + PaβN ^c	-	4	-	-	0.06	-	-	-	-	-	-	0.016	2	0.25	-	-	-
13047ΔECL_RS15545 (<i>ramR</i>) + PaβN ^c	-	8	-	-	0.125	-	-	-	-	-	-	0.03	2(-8)	0.25(-8)	-	-	-
FY01 + PaβN ^c	-	16	-	-	64	-	-	-	-	-	-	2	2	0.25	-	-	-
FY10 + PaβN ^c	-	16	-	-	64	-	-	-	-	-	-	4(-8)	2(-16)	0.5(-4)	-	-	-

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Abbreviations: PTZ: piperacillin/tazobactam; TEM: Temocillin; CAZ: Ceftazidime; CTX: Cefotaxime; FEP: Cefepime; AZT: Aztreonam; ERT: Ertapenem; MER: Meropenem; IMP: Imipenem; AMK: Amikacin; GEN: Gentamicin; CIP: Ciprofloxacin; CHL: Chloramphenicol; TIG: Tigecycline; CS: Colistin; CHX: Chlorhexidine, CTAB: Cetyl trimethyl ammonium chloride, PaβN Phe-Arg-β-naphthylamide

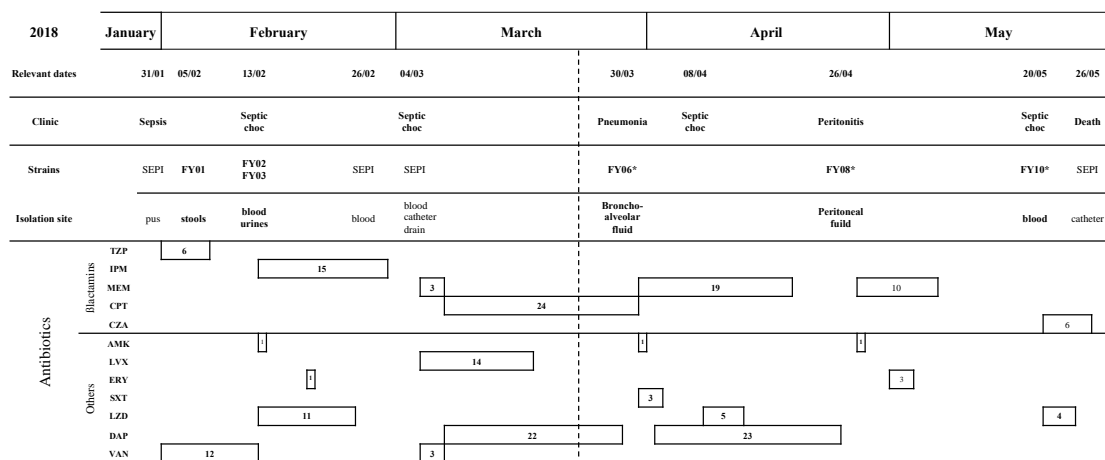
Remarks: ^a truncated *ramR*, ^b strain with ECL_RS15545 truncated gene, ^c PaβN used in a concentration of 40 mg/L

Numbers in boldface are MICs which significantly evolved: fold changes ≥ 4 or ≤ -4 . Fold changes are indicated in parentheses.

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418**Table 2.** Strains and plasmid used in this study:

Strains or plasmid	Characteristic(s)	Reference/Biosample
Strains		
<i>E. cloacae</i> ATCC13047	Reference Strain	Ren <i>et al.</i> 2010
<i>E. cloacae</i> ATCC13047ΔECL_RS15545 (<i>ramR</i>)	Deleted ECL_RS15545	This study
13047_pBAD202	13047 strain carrying pBAD202	This study
13047_pBAD202ΩECL_RS15545	13047 carrying pBAD202/D-TOPOECL_RS15545 hyperexpress <i>ramR</i> with its own promoter	This study
<i>E. cloacae</i> ATCC13047ΔECL_RS15545_pBAD202	Deleted ECL_RS15545 strain carrying pBAD202	This study
<i>E. cloacae</i> ATCC13047ΔECL_RS15545_pBAD202 ΩFY08_ <i>ramR</i> ^a	Deleted ECL_RS15545 strain carrying pBAD202 hyperexpress <i>ramR</i> with its own promoter	This study
FY01	Clinical isolate (Stools) (Date 02/05/2018)	SAMN14547852
FY02	Clinical isolate (Blood culture) (02/13/2018)	SAMN14548158
FY03	Clinical isolate (Urines) (02/13/2018)	SAMN14548159
FY06	Clinical isolate (Bronchoalveolar fluid) (03/30/2018)	SAMN14548185
FY08	Clinical isolate (Peritoneal liquid) (04/06/2018)	SAMN14548186
FY10	Clinical isolate (Blood culture) (05/20/2018)	SAMN14548187
FY01 pBAD202	ECLJO_031 strain carrying pBAD202	This study
FY01 pBAD202ΩECL_RS15545	ECLJO_031 strain carrying pBAD202/D-TOPOECL_RS15545 hyperexpress <i>ramR</i> with its own promoter	This study
FY10 pBAD202	ECLJO_031 strain carrying pBAD202	This study
FY10 pBAD202ΩECL_RS15545	ECLJO_031 strain carrying pBAD202/D-TOPOECL_RS15545 hyperexpress <i>ramR</i> with its own promoter	This study
Plasmid		
pBAD202	General expression vector with arabinose-inducible promoter, Kanamycin	Life Technologies
pKD4	Plasmid containing an FRT-flanked kanamycin cassette, Kanr	Dasenko <i>et al</i>
pCP20-Gm	FLP-mediated recombination vector, Genr	Doublet <i>et al</i>
pKOBEG	Recombination vector, phage λ <i>recyβa</i> operon under the control of the pBAD promoter, Cmr	Chaveroche <i>et al</i>

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420^a*ramR* : sequence *ramR* from FY08



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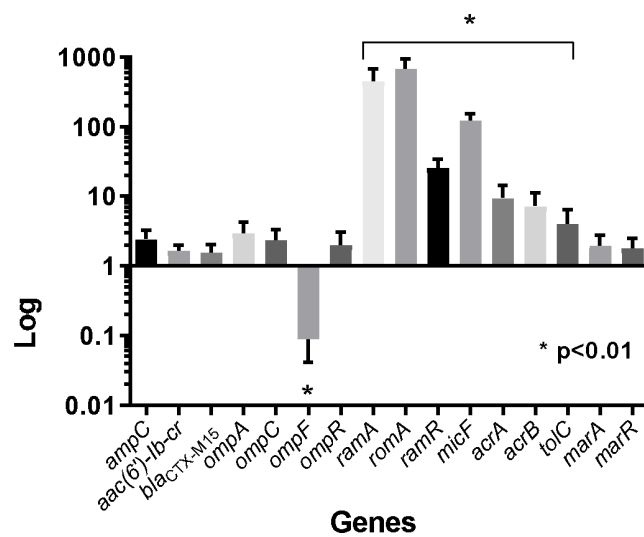
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Figure 1: Timeline of patient's hospitalization. Infectious clinical history, bacterial strains isolated and antibiotics treatments are presented. For each antibiotic duration treatments in days are indicated. The dotted line represents the apparition of the 16 bp deletion in *ramR* among the *E. hormachei* clinical strains. Abbreviations: SEPI *Staphylococcus epidermidis*, FY *E. hormachei* clinical strains, TZP piperacillin-tazobactam, IPM imipenem, MEM meropenem, CPT ceftaroline, CZA ceftazidime-avibactam, AMK amikacin, LVX levofloxacin, ERY Erythromycin, SXT trimethoprim-sulfamethoxazole, LZD linezolid, DAP daptomycin, VAN Vancomycin.

Remarks * *E. hormachei* clinical strains with an cross-resistance phenotype



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430 **Figure 2:** Differences of gene expression between strains FY06 to FY10 (group II) and FY01 to FY03 (group I) represented by bar
 431 chart. Fold changes were calculated using the delta-delta Cycle threshold (CT) method using *rpoB* as housekeeping gene. Error bar
 432 represent one standard deviation. Statistical analyses have been performed using the Student's Test on GraphPad Prism version 8.
 433 Significant p-value was considered lower than 0,05. This figure highlights that partial deletion of *ramR* led to the overexpression of
 434 seven genes *ramR*, *romA*, *ramA*, *micF*, *acrA*, *acrB* and *tolC* whereas *ompF* was downregulated.

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