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

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

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ABSTRACT

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

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INTRODUCTION:

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

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

RESULTS:

Antibiotics and antiseptics susceptibilities:

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

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

In silico typing:

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

Antibiotics resistance genetic determinants:

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

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

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

Transcriptional consequences of *ramR* deletions:

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

***Enterobacter clocacae* ATCC 13047 mutant analyzes:**

To decipher the resistance mechanism in the *E. hormaechei* strains, one mutant of *E. cloacae* ATCC 13047 deleted for the gene ECL_RS15545 was constructed. Thus, MICs of CHL, TIG and CIP were determined for the Δ*ramR* (ATCC13047ΔECL_RS15545) strain. *E. cloacae* ATCC 13047 cells in which *ramR* was deleted became significantly more resistant (FC ≥4) to CHL, TIG and CIP like the clinical strains (FY06 to FY10) harboring a 16 pb deletion in *ramR*

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(Table 1). Furthermore, the transformation of the ATCC13047ΔECL_RS15545 with the pBAD202 plasmid containing the *ramR* sequence of FY08 did not allow any restoration of the MICs (Table 1). In order to further analyze the role of the RamR regulator, two clinical isolates (FY01 and FY10) were transformed with the pBAD202 vector containing *ramR* cloned with its promoter region (Table 1). The MICs of CHL, TIG and CIP for FY10 complemented strains decreased reaching the same values as the FY01 strain (Table 1). Finally, in the presence of Phe-Arg-β-naphthylamide (PAβN), there was a significant decrease in the MICs of CHL (FC: 8), TIG (FC: 8) and CIP (FC: 4) for the ATCC13047ΔECL_RS15545 and FY10 strains, suggesting the involvement of RND efflux pumps such as AcrAB-TolC (Table 1).

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

DISCUSSION:

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

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located upstream his gene, near by the marbox could alter the RamA self-regulation (11) but there was no alteration in this region among the clinical strains (data not show). Secondly, RamA could be over-expressed by the non-functionality of RamR (12, 14). RamR is composed by two functional regions: the N-terminal one corresponding to the helix-turn-helix (HTH) domain which is the DNA binding site whereas the second one is required to the substrate recognition and binding (11, 15). In our study, RamR lost its first 69 N-Terminal amino-acids. As a consequence, RamR could not bind the DNA and could not inhibit RamA expression. To the best of our knowledge, *ramR* mutations conferring antibiotic resistance has not been described among *E. hormaechei* strains yet. However, these events have already been described among *K. pneumoniae*, *S. enterica* serovar Typhimurium and *K. aerogenes* (11–13) with a variety of mutations, which could allow bacteria to quickly adapt to antibiotics pressure (11). The *E. hormaechei* multi-resistant clinical strains may have been selected by ßlactam and fluoroquinolones treatments which were given during the February and March (Figure 1).

Results of the qRT-PCR confirmed that, a non-functional RamR lead to over-expression RamA, AcrA, AcrB, TolC, and a reduce expression of OmpF as it has been previously described (15). However, our results suggest that RamA over expression did not lead to a MarA increase expression contrarily to what has been published in *K. aerogenes* (10). Moreover, it seems the N-terminal deletion of RamR leads to its own over expression which has been inconstantly described among *K. aerogenes* clinical and mutant strains (11). Taken together these results suggest that RamR could contribute to the regulation of its own expression.

The role played by the pump AcrAB-TolC in the increase of the MICs of CHL, TIG and CIP have been demonstrated by using the PaβN (Table 1). Concerning the OmpF downregulation, MICs of the β-lactam in the ATCC13047ΔECL_RS15545 strain did not change as it was among

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

CONCLUSION:

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

METHODS

Bacterial strains

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

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

Antimicrobial agents susceptibility test

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

DNA sequencing and bioinformatics

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

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

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

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

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

Gene expression by RNA quantification

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

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

Ethics:

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

Data availability:

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

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

CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare

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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
E. cloacae 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 (*ramR*)	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_pBAD202ΩECL_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_*ramR*[a]	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_pBAD202ΩECL_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_pBAD202ΩECL_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
E. cloacae ATCC13047 + PaβN[c]	-	4	-	-	0.06	-	-	-	-	-	-	0.016	2	0.25	-	-	-
13047ΔECL_RS15545 (*ramR*) + 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)**	-	-	-

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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Table 2. Strains and plasmid used in this study:

Strains or plasmid	Characteristic(s)	Reference/Biosample
Strains		
E. cloacae ATCC13047	Reference Strain	Ren *et al.* 2010
E. cloacae ATCC13047ΔECL_RS15545 (*ramR*)	Deleted ECL_RS15545	This study
13047_pBAD202	13047 strain carrying pBAD202	This study
13047_pBAD202ΩECL_RS15545	13047 carrying pBAD202/D-TOPOΩECL_RS15545 hyperexpress *ramR* with its own promotor	This study
E. cloacae ATCC13047ΔECL_RS15545_pBAD202	Deleted ECL_RS15545 strain carrying pBAD202	This study
E. cloacae ATCC13047ΔECL_RS15545_pBAD202 ΩFY08_*ramR*[a]	Deleted ECL_RS15545 strain carrying pBAD202 hyperexpress *ramR* with its own promotor	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-TOPOΩECL_RS15545 hyperexpress *ramR* with its own promotor	This study
FY10 pBAD202	ECLJO_031 strain carrying pBAD202	This study
FY10 pBAD202ΩECL_RS15545	ECLJO_031 strain carrying pBAD202/D-TOPOΩECL_RS15545 hyperexpress *ramR* with its own promotor	This study
Plasmid		
pBAD202	General expression vector with arabinose-inducible promoter, Kanamycin	Life Technologies
pKD4	Plasmid containing an FRT-flanked kanamycin cassette, Kanr	Dasentko et *al*
pCP20-Gm	FLP-mediated recombination vector, Genr	Doublet et *al*
pKOBEG	Recombination vector, phage λ recγβα operon under the control of the pBAD promoter, Cmr	Chaveroche et *al*

[a]*ramR* : sequence *ramR* from FY08

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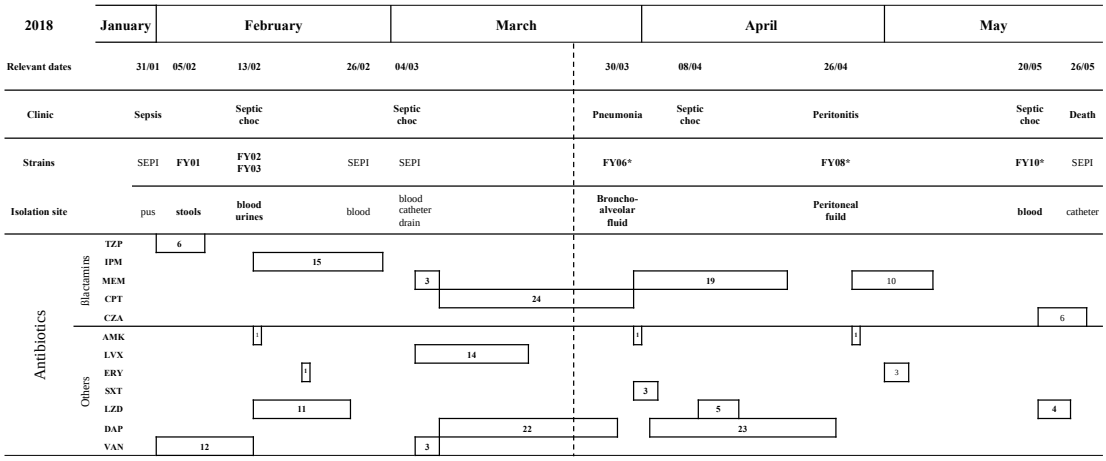

Figure 1: Timeline of patient's hospitalization. Infectious clinical history, bacterial strains isolated and antibiotics treatments are presented. For each antibiotic duration treatements in days are indicated. The dotted line represents the apparition of the 16 bp deletion in *ramR* among the *E. hormachei* clinical strains. Abbreviations: SEPI *Staphylococcus epidermidis*, FY *E. hormaechei* 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. hormaechei* clinical strains with an cross-resistance phenotype

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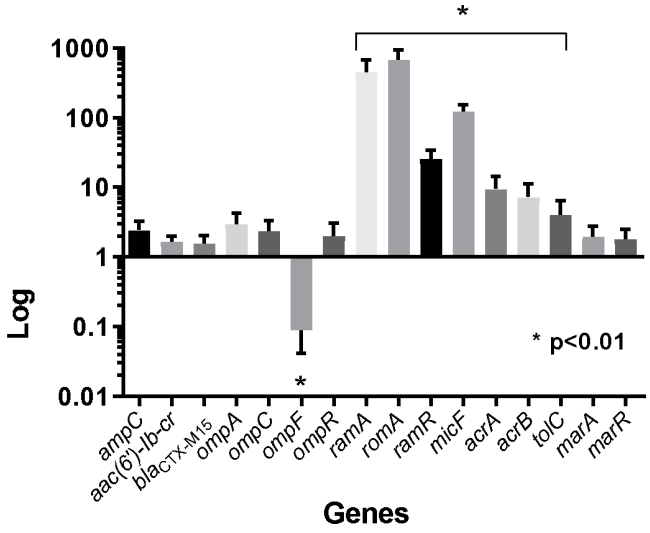

Figure 2: Differences of gene expression between strains FY06 to FY10 (group II) and FY01 to FY03 (group I) represented by bar chart. Fold changes were calculated using the delta-delta Cycle threshold (CT) method using *rpoB* as housekeeping gene. Error bar represent one standard deviation. Statistical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