Ram locus is a key regulator to trigger multidrug resistance in *Enterobacter aerogenes*

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Abstract

Purpose

Several genetic regulators belonging to AraC family are involved in the emergence of MDR isolates of *E. aerogenes* due to alterations in membrane permeability. Compared with the genetic regulator Mar, RamA may be more relevant towards the emergence of antibiotic resistance.

Methodology

Focusing on the global regulators, Mar and Ram, we compared the amino acid sequences of the Ram repressor in 59 clinical isolates and laboratory strains of *E. aerogenes.* Sequence types were associated with their corresponding Multi-drug resistance phenotypes and membrane protein expression profiles using MIC and immunoblot assays. Quantitative gene expression analysis of the different regulators and their targets (porins and efflux pump components) were performed.

Results

In the majority of the MDR isolates tested, *ramR* and a region upstream of *ramA* were mutated but *marR* or *marA* were unchanged. Expression and cloning experiments highlighted the involvement of the *ram* locus in the modification of membrane permeability. Overexpression of RamA lead to decreased porin production and increased expression of efflux pump components, whereas overexpression of RamR had the opposite effects.

Conclusion

Mutations or deletions in *ramR*, leading to the overexpression of RamA predominated in clinical MDR *E. aerogenes* isolates andwere associated with a higher-level of expression of efflux pump components. It was hypothesised that mutations in *ramR*, and the self-regulating region proximal to *ramA*, probably altered the binding properties of the RamR repressor;

thereby producing the MDR phenotype. Consequently, mutability of RamR may play a key role in predisposing *E. aerogenes* towards the emergence of a MDR phenotype.

Abbreviations : Multidrug-Resistant (MDR); Extended-Spectrum β-Lactamase (ESBL); Minimum Inhibitory Concentration (MICs); Luria-Bertani (LB); 5-Bromo-4-chloro-3-indolyl phosphate (BCIP); nitro blue tetrazolium (NBT)

Introduction

The worldwide emergence of Multidrug-Resistant (MDR) Gram-negative bacteria is a continuous health problem. This phenomenon is associated with the dissemination of selected clones of MDR bacteria as well as the local genetic adaptation of bacteria under the pressure of antibiotic exposure. Moreover, non-specific resistance mechanisms, such as the modification of membrane permeability, facilitate cross-resistance to unrelated molecules and favor the acquisition of specific resistance mechanisms such as target gene mutations and/or activation of hydrolytic enzymes, resulting in high-level drug resistance [1-3].

Enterobacter aerogenes has been recognised as a causative agent of nosocomial infection outbreaks since 1993, particularly in the Western Europe. This development was mainly due to the dispersion of an epidemic clone [3]. This event corresponded with the international spread of the Extended-Spectrum β-Lactamase (ESBL) TEM-24 ($bla_{\text{TEM-24}}$), located on an epidemic plasmid [4-5]. Infections caused by this prevalent clone are often undetected at an early stage and consequently their control and treatment have been difficult. Since 2003, *E. aerogenes* has been considered as an important MDR pathogen, particularly in intensive care units [2]. This observation has been associated with a reduced susceptibility to the most recently developed cephalosporins, including cefepime and cefpirome, and to carbapenems [6]. The alteration of envelope permeability by downregulation of porin expression, modification of lipopolysaccharides, and variation in efflux pump expression levels, has been reported to influence both virulence and strain susceptibility to various compounds [3, 7, 8]. Such adaptations were particularly observed in clinical strains isolated during carbapenem treatment of infections [6, 9]. A longitudinal study of clinical *E. aerogenes* isolates, collected over an eight-year period, indicated an important role for efflux mechanisms in the emergence of resistance [10]. The efflux pump AcrAB-TolC, identified in *E. aerogenes* clinical isolates, extrudes a variety of compounds including detergents and structurally unrelated antimicrobial

agents such as quinolones, tetracyclines, and chloramphenicol [2]. A strong correlation between AcrAB expression and the genetic regulator RamA was reported previously in *E. aerogenes* [11]. The same pattern was documented in *Klebsiella pneumoniae, Enterobacter cloacae* and *Salmonella enterica.* [12-16]. Multiple genes and external factors are involved in the emergence of MDR isolates of these three species [15, 17, 18]. This capacity for development of antibiotic resistance has been associated with a regulatory cascade involving the regulators of the AraC family (MarA, SoxS, Rob and RamA) that control the expression of membrane transporters [19, 20]. RamA seems to be more relevant to the development of antibiotic resistance in *K. pneumoniae* and *Salmonella* spp. [20-24]. Key regulatory features of the *ramA* locus are conserved amongst *Klebsiella*, *Enterobacter*, *Citrobacter* and *Salmonella* spp [21]. Modification in the expression of global activators can be mediated by mutations or ligand-mediated interactions with the cognate repressor. Various compounds such as salicylate, imipenem or chloramphenicol for *marA* and chlorpromazine, thioridazine, fluoroquinolones, cefoxitin or paraquat for *ramA* contribute to the MDR phenotype [20, 25]. In addition, several mutations located in *marRAB, ramAR, rob* or *soxRS* in clinical isolates are associated with up- and down-regulation of efflux-pump genes; the same pattern has been reported for porin synthesis, respectively [22, 26-30]. This phenomenon has been observed both *in vitro* during culture of bacteria in the presence of drugs and *in vivo* during antibiotic treatment of infected patients [31-33].

Mutations arising in specific repressors, MarR and RamR, have commonly been reported to modulate the permeability barrier in bacteria. Resolution of the MarR crystal structure confirmed that it acts as a dimer, which is a common trait of bacterial regulators [34]. Three regions of the MarR repressor are important for its activity: two putative helix-turn-helix DNA-binding domains and the first 31 amino acids, which are involved in the dimerization process [35]. Numerous mutations described in several clinical isolates of *E. coli* are scattered

105 throughout the MarR sequence, and the minimal sequence necessary for function and
106 specificity has not been defined [36]. RamR is a 191 amino acid regulatory protein in which
107 the 50 first amino acids correspond to the DNA-binding domain and the C-terminus is
108 composed of six α-helices, including important regions for dimerization. It belongs to the
109 TetR-family of transcriptional repressors and acts as a dimer on the operator region via
110 palindromic binding sites [20, 21]. Different mutations in *ramR*, have been confirmed to play
111 a role in resistance in *Klebsiella* and *Salmonella* spp. Such mutations include deletions that
112 create a premature stop site, resulting in a truncated protein; or other nucleotide deletions in
113 the putative binding site upstream of *ramA* [23, 28, 30, 37-39]. Despite these reports the true
114 clinical role of MarA and RamA in the emergence and dissemination of MDR *Enterobacter*
115 strains may be under-estimated due to the limited number of complete clinical investigations
116 to date. For the most part, only partial and case by case studies have been carried out in this
117 regard [10].

118 In this study, we characterized the sequence and function of the RamR repressor of multiple
119 clinical isolates of *E. aerogenes*. We compared the RamR amino acid sequences of 47
120 documented MDR clinical strains; 10 strains selected *in vitro* using defined antibiotics; and 2
121 reference strains. Variations in the sequence were identified and their corresponding
122 relationship(s) with the MDR phenotype was investigated in clinical isolates using MIC
123 assays for structurally unrelated antibiotics. Quantitative gene expression of the different
124 regulators and their targets pointed to a key role for RamA in the development of MDR *E.*
125 *aerogenes*. We report differences in antibiotic susceptibility and expression of porins and
126 efflux pump components among *E. aerogenes* strains that overexpress *ramA* or *ramR*. These
127 results support the working hypothesis that the *ramRA* regulon is a key player in control of
128 membrane permeability in *Enterobacter* spp.
129

Material and Methods

Bacterial strains

Fifty nine *E. aerogenes* strains were investigated: 2 laboratory reference strains, laboratory induced mutants by treatment with chloramphenicol (CM64) and imipenem (IPM5 to IPM240), and 47 clinical isolates (**Table 1)**. All strain characteristics concerning antibiotic susceptibility, outer membrane protein content and activity of an efflux mechanism have been described previously [6, 10, 11, 29]. *E. coli* strain JM109 was used for all cloning experiments and expression vector construction. All bacteria were cultivated at 37°C in Luria-Bertani (LB) Medium.

Sequencing of *marA*, *marR*, *ramA* and *ramR*

DNA from each bacterial isolate was prepared using the Wizard Genomic DNA Purification Kit (Promega). Purified DNA was then used as a template for PCR and later on for DNA sequencing to detect the presence of mutations in *marA*, *marR*, *ramA*, *ramR* and their flanking regions. The sequences of the primers are shown in **Supplementary Tables**. A final concentration of 0.4 µM of each primer was used together with, 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$, 1x *Taq* buffer and 3 units of *Taq* DNA polymerase (Qiagen). After 5 min of denaturation at 94°C, amplification was performed over 33 cycles with steps of 30 s at 94°C, 1 min at 64°C, 1 min at 72°C. A final extension step was performed at 72°C for 7 min. Amplicons were sent for nucleotide sequencing to Cogenics Online (https://www.cogenics online.com/COL/uwa.maya.engine.MayaEngine?siteid=col&mapid=home). Mutations in the amplified regions were identified by sequence alignments using BLASTN (http://www.ncbi.nlm.nih.gov /blast/Blast.cgi?PAGE=Nucleotides&PROGRAM=blastn) and CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/ index.html).

Selection of bacterial mutants by incubation with antibiotics

LB-agar-plates with increasing concentrations of chloramphenicol, rifampicin and fosfomycin were prepared. Two-fold dilution series of each antibiotic were prepared, using concentration ranges of 2-64 µg for chloramphenicol and fosfomycin ml^{-1} and 1-16 µg for rifampicin ml^{-1}, according to the Société Française de Microbiologie (http://www.sfm.asso.fr). For each antibiotic, 12 colonies of the laboratory strain ATCC13048 were picked and grown under increasing antibiotic concentrations for 24 h at 37°C.

The *ramA* and *ramR* genes of the surviving strains at the highest concentration of chloramphenicol (64 µg ml^{-1}), fosfomycin (64 µg ml^{-1}), and rifampicin (16 µg ml^{-1}) were sequenced (GenomicExpress), and compared to identify the mutations that occurred during antibiotic treatment.

Quantitative Realtime-PCR

Two or three primer pairs were designed to quantify the expression of each gene of interest , which included: *ramA* and *ramR* as representatives of the *ram*-regulon; *marA* and *marR* as key players in the *mar*-regulon; *soxR* an important gene involved in the oxidative stress regulon [24]; *acrA* as a representative component of the efflux pump [33] and *omp35* as the gene encoding the major porin involved in antibiotic influx [7, 9]. Each primer pair (**Supplementary tables**) was tested at an annealing temperature of 60°C using a standard PCR protocol to determine the optimal working primer pairs. RNA for quantitative Realtime-PCR was extracted with the RiboPure^{TM}-Yeast kit (Ambion), and quantified using a NanoDrop spectrophotometer. Contaminating genomic DNA was eliminated by two DNase I treatments according to the manufacturer's instructions (Ambion), and its absence was confirmed by including a reverse transcriptase-minus control for each RNA sample. An Eppendorf epMotion 5070 robot was used to set up the plates and the qRT-PCR was performed using an Eppendorf Mastercycler ep *realplex* Thermal Cycler.

QuantiTect SYBR Green RT-PCR (Qiagen) was used with a final concentration of 0.5 μM of each primer and 500 ng of the template RNA. After 30 min at 50°C for the reverse transcription, the HotStarTaq DNA Polymerase (Qiagen) was activated by a heating step at 95°C for 15 min. The 3-step cycles of 15 s at 94°C for denaturation, 30 s at 60°C for Annealing and 30 s at 72°C for Extension were repeated for 35-45 cycles. Each quantitative realtime PCR was repeated three times.

Expression levels of the multi-drug efflux transporter and transcriptional regulator encoding genes were compared and normalized to those of the 16S rRNA housekeeping gene. Relative levels of gene expression, compared to the ATCC13048 strain, were quantified using the 2(-Delta Delta C(T)) method of Livak and Schmittgen [40]. An increase or a decrease of 2-fold of was defined as a significant effect.

Cloning and expression of *ramA* and *ramR*

The *ramA* loci (including the putative *marbox)* and *ramR* genes were cloned from characterized strains of *E. aerogenes*. The ATCC 13048 *ramA* and *ramR* were used as an example of genes from susceptible strains, whilst EA27 (*ramA27*, *ramR27*) and CM64 (*ramA64*) were representative of MDR clinical isolates. The CM64 *ramA64* gene was identical to the ATCC13048 *ramA* (data not shown) and therefore was not cloned for this investigation. The CM64 *ramR64* gene was the only *ramR* showing a deletion at position 154/155 and therefore was used to characterize potential effects of this mutation.

All *ramA* and *ramR* genes and associated *marboxes* were amplified by PCR using primers that incorporated restriction sites at each end (detailed in **Supplementary Tables)**.

PrimeStar^{TM}HS DNA Polymerase (Takara) was used to amplify products by PCR according to the manufacturer instructions. Purified PCR-products were digested using *XhoI* and *SacI* (*ramR*) or *BamHI* and *EcoRI* (*ramA*) (New England Biolabs) and cloned into the expression vector pDrive (3851 bp) (Qiagen), using T4 ligase (NEB) to create pDrive*ramA-ATCC13048,*

pDriver*ramA-EA27*, pDriver*ramR-ATCC13048*, pDriver*ramR-EA27*, and pDriver*ramR-CM64*. Plasmid constructs were transformed into electrocompetent *E. coli* JM109 strain. Plasmid constructs were purified and confirmed by sequencing (GenomeExpress), using the primer pair T7 and SP6 (Eurogentec). Each of the 5 plasmids was then transformed separately into *E. aerogenes* ATCC13048 (representative of non-MDR strains); EA289 (a kanamycin susceptible derivative of EA27, that represented more than 90% of MDR clinical isolates); CM64 (a laboratory mutant chosen for the specific deletion in RamR at positions 154/155), and IPM240 (a laboratory strain sequentially exposed to increasing imipenem concentrations and possessing *ramA* and *ramR* genotypes that were associated with a MDR phenotype) [6]. Bacterial transformants carrying the pDriveIV plasmid were grown in the presence of antibiotics: *E. coli* with 100 μg ml^{-1} of ampicillin (Sigma) and *E. aerogenes* with 50 μg ml^{-1} kanamycin (Sigma).

Minimal inhibitory concentration (MIC) determination by E-Test stripes

ATCC13048, EA289, CM64, and IPM24 plasmid-containing strains were grown to OD_{600} 0.4 in LB containing appropriate antibiotics and then induced with IPTG (1 mM) for 1 h at 37°C. Bacteria were then sub-cultured into fresh broth and grown to OD_{623} 0.35 (approximately 10 x 10^8 colony forming units ml^{-1}). Induced cultures were diluted to 10 x 10^6 c.f.u ml^{-1}, and spread (2.5 ml) on LB Agar-plates containing 0.5 mM IPTG. After drying for several minutes, E-Test stripes were placed on the plates and bacteria grew in presence of the tested antibiotics (ciprofloxacin, norfloxacin, nalidixic acid, tetracycline, chloramphenicol, imipenem, cefoxitin, cefuroxime, cefepime and ceftazidime) over night at 37°C. Assays were independently repeated 3 times.

SDS-PAGE and Western Blotting

Bacterial protein extracts were analyzed by SDS-PAGE using 10% acrylamide. Samples were denatured in Laemmli loading dye containing 2% SDS and the protein-samples were heated three times at 95°C. Protein size was estimated by comparison with pre-stained low-range molecular weight marker (BioRad). Proteins were stained using Coomassie Brilliant Blue R-250 as previously described [6].

For immunodetection, proteins were electro-transferred onto nitrocellulose membranes (Schleicher & Schull Bioscience Inc, NH, USA) in transfer buffer (20 mM Tris, 150 mM glycine, 20% isopropanol, 0.05% SDS). Membranes were blocked using 4% milk in Tris-buffered sodium (TBS: 50 mM Tris-HCl, 150 mM NaCl, pH8). Polyclonal antibodies (Neosystem Co. Strasbourg, France), directed against denatured proteins (i.e., AcrA, AcrB and TolC), were used for detection [6, 10, 11]. Quantitation of the antigen-antibody complexes was performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, PA, USA) using BCIP and NBT (Sigma) according to the manufacturer instructions [11].

Results

Variability in regulators involved in resistance of clinical isolates

The *marA, marR* and *ramA* gene sequences of all *E. aerogenes* strains, investigated for this study, were identical to those of the susceptible ATCC13048 strain. However, 93.6 % (44/47) of the MDR clinical isolates, showed a deletion in the upstream region of *ramA,* proximal to the *marbox* (**Fig. 1**).

Several amino acid-level changes were detected in the *ramR* sequences of MDR clinical isolates compared to laboratory strains. The majority of clinical isolates (97.8%; 93.6% and 97.8% respectively) exhibited substitutions at positions 72 (Ala to Asp); 100 (Pro to Ser) and

121 (Ile to Ser). Compared with the ATCC13048 strain 93.6% (44/47) of the clinical isolates also contained an altered C-terminus, corresponding to a four amino-acid deletion. These mutations were located outside the putative DNA-binding area that corresponds to the 50 first N-terminal amino acid. Such mutations may alter the three dimensional structure of the repressor, taking into account the type of substituted side chains that have different charges and sizes compared to the original [41]. Stepwise increasing concentrations of chloramphenicol were used to select for the resistant laboratory strain CM64. When analysed, the sequence of its *ramR64* gene showed unique amino acid deletions at positions 154 (Leu) and 155 (Phe) [8]. No other strain showed such deletion.

In vitro selection of mutants can affect *ramR* structure and function.

To determine whether the characteristic deletion at position 154/155 in *ramR64* was reproducible, the ATCC13048 strain was grown under the same increasing chloramphenicol concentrations as were used to create CM64. For further verification and to determine whether new mutations would emerge, rifampicin and fosfomycin were also used in the same way; to select for resistant mutants. These antibiotics (chloramphenicol, rifampicin, and fosfomycin) are known for their capacity to select mutants at high frequency. The *ramA* and *ramR* genes of mutants, surviving sequential increasing antibiotic treatments, were sequenced. All tested strains produced mutants that survived stepwise treatment with high concentrations of rifampicin (up to 16 µg ml^{-1}) and fosfomycin (up to 64 µg ml^{-1}), and 67 % (8/12) produced mutants that survived exposure to high levels of chloramphenicol (up to 64 µg ml^{-1}). None of the fosfomycin or rifampicin resistant mutants showed variation in *ramA* or *ramR* sequences compared to the ancestral strains (data not shown).. One of the eight chloramphenicol selected mutants (CM64new-10) harbored an interesting mutation in *ramR*, which introduced a stop-codon at position 27 (**Fig. 2**).

Characterization and validation of expression of regulators of the MDR phenotype by qRT-PCR

Realtime qPCR was used to compare the levels of expression of key genes involved in *E. aerogenes* responses to antibiotic treatments and involved in the Mar and Ram regulation cascades (Table 2). The clinical isolate RAB76089G and the two imipenem resistant strains, IPM20 and IPM240, did not exhibit any significant change in expression profile compared to the reference ATCC13048 strain for all target genes (*marA, marR, ramA, ramR, soxR, acrA* and *ompA*): with the exception of RAB76089G, that showed a 2.88 fold decrease in *marR* expression (**Table 2**). All other clinical isolates (EA27, EA117, EA3, EA5, GIM55621, GIM59704, MOK75586 and PAP12515) and the laboratory mutant CM64 showed a marked increase in the expression of the transcriptional activator, *ramA* ranging from > 14-fold increase (PAP12515) to > 140-fold increase for the chloramphenicol mutant CM64. The putative repressor of the *ram*-regulon, *ramR*, was also highly expressed by clinical isoales compared to the reference ATCC13048 strain, but *ramR* expression was considerably weaker than *ramA* expression in the same isolate. Relative *ramA* expression levels ranged from > 2-fold (GIM59704) to > 10-fold (CM64). The exception was EA5 that showed > 3-fold decreased expression of *ramR* compared to ATCC13048.Three clinical isolates (EA117, EA3 and EA5) exhibited significantly increased expression of the *mar* regulon genes(*marA* and *marR*), and *soxR* (ranging between> 20-fold to >160-fold increase).The remaining strains showed only marginal variations in expression that were not significantly different to the reference strain.

The majority of MDR clinical isolates were found to be upregulating expression of the efflux pump component *acrA* (up to a 3-fold increase) and downregulating expression of the major porin gene *omp35* (down to a 9-fold decrease). IPM20 and IPM240 mutants, selected using sequentially increased concentrations of imipenem, showed similar expression of the tested

genes to the reference ATCC13048 strain. This can be explained by the lack of imipenem selective pressure used during growth of these cultures for the qRT-PCR experiments.

Overexpression of *ramA* or *ramR* can regulate the expression of efflux-pump components and outer membrane porins

Construction of strains that over-expressed *ramA* or *ramR*, and immune-detection of key membrane permeability components, corroborated the associated variation observed in gene expression at the protein level. Western blot analysis showed significant variations in the expression of major porins (Omp35 and Omp36) and components of efflux pumps (AcrA, AcrB, TolC) in several strains that over-expressed *ramA* or *ramR*. A notable decrease in porin protein expression was observed in protein extracts from transformed strain EA27 that over-expressed *ramA* from ATCC13048 and EA27 By contrast, when the same strain was modified to over-express *ramR* from ATCC13048, EA27 and CM64 a small increase in porin protein expression was observed (Figure 3). At the same time, no variation of the OmpA, an outer membrane protein involved in the membrane organization, was observed [2]. Similar effects were observed for strains EA289, CM64, and IPM240 that over-expressed *ramA* (data not shown). Overexpression of *ramR* in ATCC13048 showed no alteration of the porin content, regardless of the *ramR* origin (data not shown).

The effects of *ram* gene over-expression on the production of the efflux pump components (AcrA, AcrB, and TolC) are also presented in **Figure 3**. Both *ramA* of ATCC13048 and EA27 induced a small increase in AcrA expression, along with AcrB and TolC in EA289, CM64 and IPM240. Overexpression of *ramR* from ATCC13048, EA27, and CM64 had the opposite effect and generated significant decreases in the signals of efflux pump components in the three tested strains. No variation of efflux pump components was observed in the strain ATCC13048 when *ramR* was overexpressed (data not shown).

Involvement of RamA overexpression on the antibiotic phenotype

Both ATCC13048 and the imipenem-selected mutants (IPM20 and IPM240) exhibited a significant change in their antibiotic susceptibility profiles when *ramA* was overexpressed. Table 3 shows that the ATCC13048 strain presented increased resistance to a to nalidixic acid tetracycline, and chloramphenicol, (6-fold higher MIC), and to cefepime (4-fold higher MIC). The strain IPM240 showed increases in resistance to nalidixic acid and tetracycline. Overexpression of *ramA* had only slight effects in already MDR-strains EA289 and CM64 (data not shown).

Involvement of *ramR* overexpression on the antibiotic phenotype of a strain selected under increasing concentration of imipenem.

E-test strip analysis of the imipenem-selected mutant IPM240, that had been modified to over-express *ramR* from 3 different sources (ATCC13048, EA27, and CM64), resulted in a nearly 3-fold decrease in the minimum inhibitory concentration of imipenem from 32 μg ml^{-1} to 12 μg ml^{-1} (**Table 4**). A significant decrease in the MICs was also observed for tetracyclin and chloramphenicol. Overexpression of *ramR* in EA289 and CM64 resulted in only minor effects and no effect at all was observed when the various *ramR* genes were over-expressed in ATCC13048 due to a native inhibition in this strain (data not shown).

Discussion

Numerous regulators have been described in the development of bacterial MDR and both structural and genetic investigations endeavor to understand and decipher their mechanisms of action [14, 18, 19]. *Enterobacteriaceae* have evolved different molecular resistance strategies in response to a variety of toxic compounds and environmental stresses by way of the membrane permeability modulation, which is associated with the expression of drug transporters including porins and efflux pumps. The control of their expression is carried

out at several levels: global or local regulators, activators, repressors and response to chemical or pharmaceutical factors [3].

One positive regulator of growing interest in *E. aerogenes* is the global transcriptional activator RamA that is known to be involved in the balance of outer membrane permeability and in the active extrusion of intracellular antibiotics. RamA shares high similarity with MarA and it can be expected that *ramA* and *marA* might recognize an overlapping set of operator sequences. Previous reports have revealed that mutations or gene interruptions could be acquired either within *ramR* or in the *ramA* promoter [23]. In the present study, sequence analysis of various MDR strains of *E. aerogenes* revealed the presence of several mutations in *ramR* that were located outside the region involved in the DNA-binding domain of TetR family repressors, they were found in the C-terminus, that was associated with protein dimerization.

These *ramR* mutations have not previously been described in *E. aerogenes* or in other related species, such as *S. enterica, K. pneumoniae,* and *E. cloacae* [22-24, 28, 33, 37, 39-42]. These mutations could induce structural changes, inactivating the RamR repressor function. The deletion upstream of *ramA* was located between the gene and the putative *marbox*, responsible for self-regulation of the gene. The binding site of RamR contains essential features of the *ramA* promoter, including the -10 conserved region, the transcriptional start site of *ramA* and two 7-bp inverted repeats [23]. Modifications here could alter protein-DNA binding and hence the self-regulation of *ramA*. Both detected modifications could therefore be responsible for the increased expression of *ramA* that consequently trigger the MDR phenotype. It has been previously demonstrated that sequence alterations in *ramR* or in the upstream region of *ramA* led to an up-regulation of AcrAB in *Salmonella enterica* [31, 43]. A characteristic deletion at position 154/155 in *ramR* was only found in a chloramphenicol-resistant mutant (CM64) that was selected for with increasing concentrations of chloramphenicol [8, 44]. A mutation in the repressor causing increased expression of the

global activator might bring benefits for bacterial survival in an otherwise hostile environment.

Expression patterns of *marA* and *marR* indicated that a balance between expression of activators and repressors takes place in *E. aerogenes* resistant isolates. An increased expression of RamR, that does not affect expression of *ramA*, indicated that the repressor would be less functional, probably as a result of the detected mutations. After growing in the absence of antibiotics, the *E. aerogenes* strains rapidly re-regulated membrane permeability and showed the same expression patterns as the susceptible reference strain ATCC13048. In contrast, the expression pattern of the chloramphenicol resistant mutant CM64, exhibiting a deletion in *ramR*, was comparable with those of the clinical isolates. The clinical isolates showing a MDR phenotype also exhibited a decreased transcription of porin gene (*omp35*) and an increased expression of the efflux pump gene, *acrA*. These results depicted a consistency between the increased expression of both *ramA* efflux pumps, and the decreased expression of porins that triggers the MDR phenotype in clinical isolates. Some MDR clinical isolates showed increased expression of *omp35* that can be balanced out by the post-transcriptional control of porin genes or the post-translational control of porin assembly into the bacterial outer membrane [8]. In contrast, the expression of *marA* remained on a comparable level with the susceptible reference strain. As suggested by Wang *et al*. in *K. pneumoniae,* MarA might serve as an alternative regulator and RamA would be the most potent regulator of the MDR phenotype [21, 42]. This observation points to the importance of the global regulator *ram* for the MDR-phenotype in *E. aerogenes*. However, Martin *et al*. compared activation of a set of promoters containing marboxes, depending of MarA or SoxS concentration [19]. They observed that the half maximal activation of promoters by MarA was highly concentration dependent, and correlations between *in vivo* and *in vitro* experiments measuring optimal activator concentration were poor, and the promoter

activation profile depends specifically on the activator. So target gene activation is thought to depend on the concentration and the nature of a given activator of the *mar* regulon.

Cloning experiments confirmed the role of RamA in reducing porin expression and increasing the expression of efflux pump components; this pattern was consistent with the altered corresponding antibiotic susceptibility profile. In *S.* Typhimurium and in *K. pneumoniae*, several studies have confirmed the role of RamA in fluoroquinolones resistance due to AcrAB overexpression [14, 15, 18, 23, 31, 36]. These results supported the assumption that RamA is a global regulator triggering the MDR phenotype by modification of the membrane permeability with *ramR* acting as its local operonic repressor. Despite identical RamR mutations found in most of the various clinical strains studied, the level of expression of *ramA* was variable and antibiotic MICs were not identical. However, it has been demonstrated that increasing *ramA* expression due to identical RamR deregulation was variable. As observed by Bailey *et al.*, bacterial carefully "orchestrate" the level of RamA, expression and genes within its regulon are produced at the correct level only under appropriate conditions [15]. This is supported by the existence of alternate pathways in *ramA* regulation as the level of other regulators and the intracellular concentration-dependent response of the bacterium to increasing overexpression of the transcriptional activator [20, 41]. Conversely, diverse mutations in RamR were able to stimulate identical *ramA* overexpression as previously demonstrated [42]. Moreover, considering the reported influence of RamA on more than 100 genes, the combination of their level of expression could result in a panel of pleiotropic MDR phenotypes [20]. Regarding the strain CM64 that presented a particular deletion in RamR, expression of *ramA* expression was increased by more than 100-fold compared to the others, suggesting that this deletion was more important in the regulation cascade. Importantly, the MDR phenotype of CM64 was not particularly different, a feature that makes it difficult to further elucidate their role in functional changes in *ramR*.

Rosenblum *et al.* observed that in *K. pneumoniae,* RamR mutations could be found in both DNA and ligand binding domains, suggesting that there were no mutational hotspots within RamR [24]. Moreover, it has been demonstrated that several mutations are required for MarR inactivation, so as to obtain a significant alteration of its repressor activity [23, 24, 30, 35, 36]. Genes regulated by RamA seemed to be controlled by it in a concentration-dependent manner, as observed by qRT-PCR experiments and Western blotting results.

The MDR phenotype in *E. aerogenes* is caused by several factors acting in concert. The combination of an enzymatic barrier caused by several antibiotic-degrading enzymes, a target-protection barrier caused by mutations in the targets of antibiotics, and the physical barrier by alteration of the outer membrane profile, work together to protect the bacteria from harmful substances. Alteration of just one of these barriers will not switch off MDR. This feature is supported by the fact, that overexpressed *ramA* or *ramR* alter the membrane profile of the tested MDR strains by increasing or decreasing the expression of porins or efflux pump components as shown by immunoblot analysis, but the *E. aerogenes* susceptibility to various antibiotics was not completely modified, as shown by MIC data. The strain EA289 remained resistant to the majority of the tested antibiotics because the enzymatic and the target protection barrier are insensitive to the overexpression of *ramA* or *ramR* [1, 3]. The reason that the chloramphenicol-selected mutant, CM64 maintained its MDR phenotype, despite over-expression of *ramR* could not be determined in this study. Since chloramphenicol induced a high mutation rate, we cannot eliminate the possibility that the CM64 mutant possesses mutations in other loci. These mutations could also contribute to the MDR phenotype. The fact that *ramR* did not have any effect on the already antibiotic susceptible strain ATCC13048 can be explained by the normal production of *ramR* in the susceptible strain. An overexpression of *ramA* on the other hand may contribute to an imbalance in the ratio between activator *ramA* and repressor *ramR*, thereby altering membrane permeability and thus increasing resistance to several antibiotics as shown by the MIC data.

To conclude, this study demonstrated that the global regulator *ram* is important in the cascade of membrane permeability. The mutations identified in *ramR* seem to modify the structure of the protein required for the affinity of the binding site as previously reported for MarR and TetR repressor family and hereby leave it less functional compared to the wild-type [25, 43]. In addition, the activity of the global regulator also depends on its expression level. Overexpression of a less functional repressor is able to modify the content of porins and efflux pumps in the outer membrane. Finally, this was the first study to provide data describing the direct correlation between the expression of genes constituting the *ram*-regulon and their respective influence on membrane permeability in *E. aerogenes*.

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Figures legend

Figure 1: Sequence upstream of *ramA* (represented by startcodon ATG) with putative *marbox* and deletion found in several clinical isolates. IPM represents the 9 strains IPM1-IPM240. EA103280 and EA112978 are non-MDR clinical isolates. Strains GIM59704, MOK75586, PAP12586, RAB73482 and EA27 represent 91.5% (43 of 47) of all tested MDR clinical isolates.

Figure 2: Comparison of the amino-acid sequence of RamR identified in 2 variants of the ATCC13048 reference strain, obtained after treatment with chloramphenicol; CM64 was characterized previously [43] and CM64 new-10 was identified form the stepwise treatment with chloramphenicol.

Figure 3: Expression of AcrA, AcrB, TolC and Omp36 in *Enterobacter aerogenes* strain EA289 harboring different plasmids. Lines 1-6: **1** empty plasmid pDriveIV, **2** insert *ramA* ATCC, **3** *ramA* EA27, **4** *ramR* ATCC, **5** *ramR* EA27 and **6** *ramR* CM64. Increased expression respectively of AcrA, AcrB, TolC due to overexpression of *ramA* marked with stars *****, decrease due to *ramR* marked with **¤.** Lowered expression of *omp36* due to overexpression of *ramA* marked with stars *****

Tables

Strains	Year of isolation/origin	MIC (mg l^{-1})				Outer membrane protein content	
		Antibiotics*					
		CHL	CIP	FEP	IMP	Porin	Efflux
ATCC 13048	Reference	16	0.25	0.25	0.125	yes	no
ATCC15038	Reference	2	0.125	ND†	0.25	yes	no
CM64	Laboratory [43]	256	2	0.5	0.25	yes	yes
EA117	1996[44]	512	256	64	0.25	yes (weak)	yes
EA119	1996[44]	16	256	32	0.125	yes (weak)	no
EA3	1996[44]	ND	ND	64	4	yes (modified)	yes
EA27	1996[44]	512	256	64	8	no	yes
EA5	1996[44]	512	256	64	4	no	yes
EA19	1996[44]	1024	>512	64	1	yes	yes
EA14	1996[44]	1024	512	32-64	2	yes	yes
EA7	1996[44]	256	64	4	1	yes	yes
EA45377	1995[10]	ND	ND	ND	ND	ND	ND
EA103	1995[10]	ND	ND	16	16	no	no
EA111	1995[10]	ND	ND	16	8	no	ND
EA110	1995[10]	ND	ND	64	16	no	ND
EA102	1995[10]	ND	ND	32	16	no	ND
EA121653	2003[10]	256	256	1	1	ND	no
EA1061701	1995[10]	>256	128	64	16	ND	yes
EA109688	2003[10]	16	128	4	1	ND	yes
EA108	1995[10]	<4	64	ND	2	ND	no
EA103280	2003[10]	8	<4	1	4	ND	yes
EA54	1995[10]	16	64	4	2	ND	yes
EA112978	2003[10]	<4	256	4	4	ND	no
EA6582	1995[10]	256	256	4	4	ND	yes
GIM63001	1997[6, 29]	256	64	4	2	yes	ND
GIM59705	1997[6, 29]	256	128	64	16	no	ND
GIM59704	1997[6, 29]	256	128	32	16	no	ND
GIM53292	1997[6, 29]	512	128	32	32	no	ND
GIM54584	1997[6, 29]	256	128	16	4	yes	ND
GIM55621	1997[6, 29]	512	128	128	8	no	ND
GIM55625	1997[6, 29]	256	64	2	1	yes	ND
GIM59627	1997[6, 29]	512	128	64	8	no	ND

MOK72691	1997[6]	256	256	128	16	no	ND
MOK73694	1997[6]	128	256	128	16	yes	ND
MOK75586	1997[6]	128	256	32	32	no	ND
MOK76500	1997[6]	128	256	2	1	yes	ND
PAP11668	1997[6, 29]	8	2	1	1	yes	ND
PAP13165	1997[6, 29]	256	64	64	16	no	ND
PAP12698	1997[6, 29]	256	32	16	2	yes	ND
PAP12586	1997[6, 29]	256	32	8	2	yes	ND
PAP12515	1997[6, 29]	256	32	64	16	no	ND
RAB73698	1997[6]	256	64	4	2	yes	ND
RAB73482	1997[6]	256	64	2	1	yes	ND
RAB76089G	1997[6]	256	64	128	8	no	ND
RAB76089P	1997[6]	512	32	2	4	yes	ND
IPM5	Laboratory	-	-	-	-	no	yes
IPM20	Laboratory	-	-	-	-	no	yes
IPM40	Laboratory	-	-	-	-	no	yes
IPM60	Laboratory	-	-	-	-	no	yes
IPM70	Laboratory	-	-	-	-	no	yes
IPM80	Laboratory	-	-	-	-	no	yes
IPM120	Laboratory	-	-	-	-	no	yes
IPM160	Laboratory	-	-	-	-	no	yes
IPM240	Laboratory	-	-	-	-	no	yes
106206	2003[10]	256	128	64	4	ND	yes
112978	2003[10]	<4	256	4	4	ND	no
131102	2003[10]	>256	256	2	4	ND	yes
131538	2003[10]	>256	256	32	8	ND	yes
137464	2003[10]	>256	512	64	8	ND	yes

Table 1: Clinical and laboratory strains studied. Data concerning MICs and outer membrane proteins content were obtained in precedent studies [6, 10, 29, 43, 44].

*CHL, chloramphenicol; CIP: ciprofloxacin; FEP: cefepime; IMP: imipenem.

†ND: not determined.

strains	genes						
	ramA	***ramR***	***marA***	***marR***	***soxR***	***acrA***	***omp35***
EA27	28.42	4.66	-1.24	1.07	-2.88	1.92	-2.75
CM64	147.80	10.90	2.98	3.39	-2.10	3.19	-4.59
IPM20	1.01	1.12	1.05	1.28	-1.00	1.06	1.13
IPM240	1.09	1.09	-1.15	-1.10	-1.38	-1.26	1.69
EA117	40.61	5.42	39.26	27.29	91.09	2.18	3.63
EA3	29.78	6.14	22.73	15.41	51.94	1.91	2.96
EA5	68.76	-3.52	54.93	52.85	169.59	3.80	9.02
GIM55621	18.07	3.03	5.01	2.84	9.25	1.64	1.03
GIM59704	19.33	2.26	-1.32	-1.37	-2.73	2.37	-2.14
MOK75586	17.62	2.28	1.02	1.02	-4.25	3.02	-2.10
PAP12515	14.37	2.49	-3.26	-2.19	-4.83	1.41	-2.31
RAB76089G	-1.81	-1.80	-1.16	-2.88	-1.42	-1.02	-1.19

Table 2: DDCt qRT-PCRs results. The values are relative to the reference strain ATCC13048.

Strains	**Antibiotic MIC (µg ml^{-1})***								
	CIP	**NFX**	**NAL**	**TET**	**CHL**	**IMP**	**CFX**	**FEP**	**CAZ**
ATCC13048									
pDrive	0.25	1	4	4	8	2	8	0.25	1.5
p*ramA*	0.5	4	24	24	48	2	16	1	3
IPM240									
pDrive	0.19	2	6	16	48	>32	---†	3	12
p*ramA*	0.25	3	12	48	48	>32	---	3	8

Table 3: Combination of strains and plasmids with corresponding antibiotic MICs in µg ml^{-1}.

*CIP, Ciprofloxacin; NFX, Norfloxacin; NAL, Nalidixic acid; TET, Tetracycline; CHL, Chloramphenicol; IMP Imipenem; CFX, Cefuroxime; FEP, Cefepime; CAZ, Ceftazidime.

†--- means no inhibition could be remarked

Strains	MIC (µg ml^{-1}) *								
	CIP	**NFX**	**NAL**	**TET**	**CHL**	**IMP**	**CFX**	**FEP**	**CAZ**
IPM240									
pDrive	0.19	2	6	16	48	>32	---†	3	12
p*ramR* ATCC	0.125	1	4	24	12	12	---	3	16
p*ramR* EA27	0.125	1	8	12	16	12	---	2	12
p*ramR* CM64	0.125	0.5	4	6	4	12	---	3	24

Table 4: Combination of strains and plasmids with corresponding MICs in µg ml^{-1}.

*CIP, Ciprofloxacin; NFX, Norfloxacin; NAL, Nalidixic acid; TET, Tetracycline; CHL, Chloramphenicol; IMP Imipenem; CFX, Cefuroxime; FEP, Cefepime; CAZ, Ceftazidime.

†--- means no inhibition could be remarked