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In Vitro Selection of *ramR* and *soxR* Mutants Overexpressing Efflux Systems by Fluoroquinolones as Well as Cefoxitin in *Klebsiella pneumoniae*▽

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The relationship between efflux system overexpression and cross-resistance to cefoxitin, quinolones, and chloramphenicol has recently been reported in *Klebsiella pneumoniae*. In 3 previously published clinical isolates and 17 *in vitro* mutants selected with cefoxitin or fluoroquinolones, mutations in the potential regulator genes of the AcrAB efflux pump (*acrR*, *ramR*, *ramA*, *marR*, *marA*, *soxR*, *soxS*, and *rob*) were searched, and their impacts on efflux-related antibiotic cross-resistance were assessed. All mutants but 1, and 2 clinical isolates, overexpressed *acrB*. No mutation was detected in the regulator genes studied among the clinical isolates and 8 of the mutants. For the 9 remaining mutants, a mutation was found in the *ramR* gene in 8 of them and in the *soxR* gene in the last one, resulting in overexpression of *ramA* and *soxS*, respectively. Transformation of the *ramR* mutants and the *soxR* mutant with the wild-type *ramR* and *soxR* genes, respectively, abolished overexpression of *acrB* and *ramA* in the *ramR* mutants and of *soxS* in the *soxR* mutant, as well as antibiotic cross-resistance. Resistance due to efflux system overexpression was demonstrated for 4 new antibiotics: cefuroxime, cefotaxime, ceftazidime, and ertapenem. This study shows that the *ramR* and *soxR* genes control the expression of efflux systems in *K. pneumoniae* and suggests the existence of efflux pumps other than AcrAB and of other loci involved in the regulation of AcrAB expression.

Klebsiella pneumoniae is an important pathogen, both in the community and in the hospital setting, and is responsible for a variety of infections, including urinary tract infections, pneumonia, liver abscesses and bacteremia (18, 34). Acquired resistance to β-lactams in this bacterial species can be due to expression of different enzymes, such as extended-spectrum β-lactamases (ESBLs) (39), plasmid-mediated AmpC β-lactamases (30), and, more recently, carbapenemases (45). Moreover, two other nonenzymatic mechanisms participate in resistance to β-lactams in *K. pneumoniae*. One of them is porin alteration, which has been involved in resistance to cefoxitin and, more recently, to ertapenem in mutants producing the ESBL CTX-M-15 (20). The second is efflux system overexpression, which has recently been shown to induce low-level cross-resistance to different antibiotic families, including quinolones, chloramphenicol, and β-lactams (28, 29). This efflux-related antibiotic cross-resistance has been observed in almost 5% of the non-plasmid-mediated β-lactamase-producing *K. pneumoniae* clinical isolates in our hospital since 2000 (29).

The efflux system most extensively studied to date is the AcrAB pump, which belongs to the resistance-nodulation-division (RND) family and is associated with the outer membrane protein TolC. AcrAB-TolC is present in various bacterial species belonging to the family *Enterobacteriaceae*, such as *Salmonella enterica*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Morganella morganii*, *Proteus mirabilis*, and *K. pneumoniae* (21, 26, 31, 33, 35, 36, 41). Substrates for this efflux system include quinolones, chloramphenicol, macrolides, tetracycline, tigecycline, trimethoprim, and β-lactams (3, 23, 32, 36, 37, 38). Besides its implication in antibiotic resistance, the participation of the AcrAB efflux system in bacterial virulence has been demonstrated in *S. enterica*, *E. coli*, and, recently, *K. pneumoniae* (3, 6, 15, 28).

More insight into the regulatory pathways that control expression of the AcrAB-TolC system in some *Enterobacteriaceae* species has been gained over the last few years. Expression of the *acrAB* operon is regulated by its local repressor, AcrR (32). At a global level, it is also influenced by several transcriptional activators belonging to the AraC/XylS family, especially MarA, RamA, SoxS, and Rob (2, 12). Transcription of the *marRAB* locus is activated by the binding of MarA to the *marO* operator region, whereas it is repressed by the binding of MarR (9, 40). More recently, the local repressor of RamA, called RamR, whose gene is found directly upstream of *ramA* in the opposite orientation, has been identified in *S. enterica* (1). SoxS is the effector of the *soxRS* global superoxide response regulon, and its expression can be activated by its regulator, SoxR, present in its oxidized form (43). It has also been shown that mutations in the regulator genes *acrR*, *marR*, *ramR*, and *soxR* can result in overexpression of the *acrAB* operon and

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TABLE 1. Primers used in this study

Primer use and target region	Primer	Oligonucleotide sequence	T_m (°C)[a]	Reference
Sequencing				
gyrA	gyrA6	5′-CGACCTTGCGAGAGAAAT-3′	56	7
	gyrA631	5′-GTTCCATCAGCCCTTCAA-3′		
parC	parC F	5′-CTGAACGCCAGCGCGAAATT-3′	58	7
	parC R	5′-TGCGGTGGAATATCGGTCGC-3′		
acrR	acrR F	5′-GCTAAGCTGCCTGAGAGCAT-3′	58	This study
	acrR R	5′-ATGCAAATGCCGGAGAATAC-3′		
ramR	ramR F	5′-CACGGTTCATATCCTGACCA-3′	60	This study
	ramR R	5′-CCRTCGACCTTAAACACGTC-3′		
ramA	ramA F	5′-TGGGATGAACCGTATCAACG-3′	58	This study
	ramA R	5′-ATCTTACTGCTGGCCCTGCT-3′		
marA-marR	loc mar F	5′-CATAGCTGAGGCTGGAGRCC-3′	56	This study
	loc mar R	5′-TCGGCCAATTCATAATGTTG-3′		
soxS-soxR	loc sox F	5′-CGGAACCTCCATCAACAGATT-3′	60	This study
	loc sox R	5′-GCAGGTAAGCTGGCTCTACAA-3′		
rob	rob F	5′-TCACGCACTTAGCAGAAAAGG-3′	60	This study
	rob R	5′-ACTATCAGCAAAGCCCGTGG-3′		
RT-PCR				
acrB	acrB F	5′-CGATAACCTGATGTACATGTCC-3′	60	10
	acrB R	5′-CCGACAACCATCAGGAAGCT-3′		
ramA	ramA F	5′-ATCGTCGAGTGGATTGATGA-3′	60	5
	ramA R	5′-AGATGCCATTTCGAATACCC-3′		
soxS	soxS F	5′-GCATCACGGTACGGAACAT-3′	60	5
	soxS R	5′-AGTCGCCAGAAAGTCAGGAT-3′		
rpoB	rpoB F	5′-AAGGCGAATCCAGCTTGTTCAGC-3′	60	10
	rpoB R	5′-TGACGTTGCATGTTCGCACCCATCA-3′		
Cloning				
ramR	ramR F	5′-CACGGTTCATATCCTGACCA-3′	60	This study
	ramR R	5′-CCRTCGACCTTAAACACGTC-3′		
soxR	soxR F	5′-AACCAGCGAGATAATGCGAA-3′	53	This study
	soxR R	5′-ATAAAGCGGCCTCTCTCAAT-3′		

contribute to a multidrug-resistant phenotype (1, 19, 24, 27, 38, 42). However, these systems have been studied mainly in *E. coli* and *S. enterica*, and little is known about their roles in *K. pneumoniae*, and the effects of mutations in the regulator genes on resistance to β-lactams are still unclear.

Therefore, the present study had a triple objective: (i) assessing the effect of antibiotic pressure by cefoxitin, on one hand, and by fluoroquinolones, on the other hand, on *in vitro* selection of resistant mutants overexpressing an efflux system from fully susceptible *K. pneumoniae* strains; (ii) searching for the presence of mutations in the regulator genes *acrR*, *ramR*, *ramA*, *marR*, *marA*, *soxR*, *soxS*, and *rob* in clinical isolates and in *in vitro*-selected mutants displaying an efflux-related antibiotic cross-resistance pattern; and (iii) determining the impacts of these mutations on the susceptibilities of the different strains to a large panel of antibiotics.

MATERIALS AND METHODS

Bacterial strains. The collection studied comprised (i) 3 previously published *K. pneumoniae* clinical isolates shown to have cross-resistance to cefoxitin, quinolones, and chloramphenicol (KPBj E+), with increased expression of the *acrB* gene for 2 of them (KPBj1 E+ and KPBj3 E+) (3, 29); (ii) their spontaneous revertants (KPBj Rev) susceptible to the three antibiotic families (3); and (iii) mutants selected from these 3 revertant strains with cefoxitin, ciprofloxacin, or levofloxacin. For all experiments, *K. pneumoniae* strain ATCC 138821 was used as a control.

***In vitro* selection of mutants.** Mutants were selected by using the procedure previously described by Miller et al. (22). Briefly, 5 μl of an overnight culture was transferred into 9 ml of fresh Mueller-Hinton (MH) broth containing ciprofloxacin (Sigma Aldrich, Saint-Quentin-Fallavier, France), levofloxacin (Sigma Aldrich), or cefoxitin (Panpharma, Fougères, France) at 0.25× MIC for the strain tested and incubated for 18 h at 37°C. Such growth conditions were repeated until mutants were obtained from overnight culture aliquots plated after each broth culture cycle on MH agar containing 4× the original MIC.

Antibiotic susceptibility. The susceptibilities of the *in vitro*-selected mutants to the well-known markers of efflux system overexpression (i.e., cefoxitin, nalidixic acid, and chloramphenicol) were determined by using the agar disk diffusion method. Only the mutants displaying cross-resistance to these 3 antibiotics were further studied. Susceptibility to a large panel of antibiotics, including β-lactams (cefoxitin, amoxicillin, and amoxicillin associated with clavulanic acid, ticarcillin, cefazolin, cefuroxime, cefotaxime, ceftazidime, ertapenem, imipenem, meropenem, and doripenem), quinolones (nalidixic acid, ciprofloxacin, and levofloxacin), chloramphenicol, tetracycline, and tigecycline, was determined by the agar dilution method. MIC determination, replicated 3 times, was performed and interpreted following the recommendations of the French Antibiogram Committee [http://www.sfm.asso.fr/nouv/general.php?pa=2].

Real-time RT-PCR for analysis of gene expression. The transcription (mRNA) levels of the *acrB*, *ramA*, and *soxS* genes were determined according to the method previously described by Doumith et al. (10). Briefly, total cellular RNA was extracted using the RNeasy Mini kit (Qiagen, Courtaboeuf, France) and treated with RNase-free DNase (Qiagen) for 30 min at 37°C, after which a second step of purification was performed. Reverse transcription (RT)-PCR was carried out in a LightCycler using the one-step LightCycler RNA Master SYBR green I kit (Roche Applied Science, Meylan, France) and the primers listed in Table 1. The specificity of the generated products was tested by melting-point analysis. Amplifications were performed in duplicate from two different RNA preparations. The cycle threshold (C_T) values of the target genes were compared with the C_T values of the housekeeping *rpoB* gene, chosen as an endogenous reference for normalizing the transcription levels of the target genes. Strain ATCC 138821 was used as a control, and the normalized relative expression of

TABLE 2. GenBank accession numbers

Strain	Accession number					
	acrR	*ramR*	*ramA*	*marA-marR*	*soxS-soxR*	*rob*
KPBj1 Rev	GU985172	HM036711	GU985178	HM010916	HM036717	HQ992823
KPBj1 E+	GU985173	HM036712	GU985179	HM010917	HM036718	HQ992824
KPBj3 Rev	GU985174	HM036713	GU985180	HM010918	HM036719	HQ992825
KPBj3 E+	GU985175	HM036714	GU985181	HM010919	HM036720	HQ992826
KPBj5 Rev	GU985176	HM036715	GU985182	HM010920	HM036721	HQ992827
KPBj5 E+	GU985177	HM036716	GU985183	HM010921	HM036722	HQ992828

the *acrB*, *ramA*, and *soxS* genes was determined for each strain according to the following formula: $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = (C_T - C_{TrpoB})_{\text{studied strain}} - (C_T - C_{TrpoB})_{\text{control strain}}$.

Analysis of gene sequences. The presence of mutations in the *gyrA* and *parC* genes, which encode targets for quinolones, and in the *acrR*, *ramR*, *ramA*, *marR*, *marA*, *soxR*, *soxS*, and *rob* genes was assessed by PCR and sequencing. The sequences of the primers used, as well as the annealing temperature (T_m), for each gene are shown in Table 1 (7). PCR was performed with a 0.2 μM concentration of each primer, a 500 μM concentration of the deoxynucleoside triphosphates (dNTPs), 1× PCR buffer with $MgCl_2$, and 2.5 U of *Taq* DNA polymerase per 50-μl reaction mixture. After a 5-min denaturation at 95°C, amplification was performed for 35 cycles of 1 min at 95°C, 1 min at T_m, and 1 min at 72°C, with a final extension of 10 min at 72°C. After purification of the PCR products on Bio-Gel P-100 polyacrylamide gel (Bio-Rad, Marne-la-Coquette, France) columns, the presequencing amplification reaction was performed with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's protocol. The PCR products obtained were then purified on Sephadex G-50 (Amersham Biosciences, Orsay, France) columns and sequenced on the 3130xl Genetic Analyzer (Applied Biosystems). The presence of mutations in the amplified regions was assessed by multiple-sequence alignments using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Nucleic acid sequences were then translated into corresponding protein sequences using the EMBOSS Transeq application (http://www.ebi.ac.uk/Tools/emboss/transeq/), in order to further analyze the consequences of the mutations observed.

Complementation with the wild-type *ramR* or *soxR* gene. The 895-bp *ramR* fragment, generated by PCR using primers RamR F and RamR R (Table 1) from the genomic DNA of strain ATCC 138821, was cloned into the pSC-A-amp/kan plasmid vector using a StrataClone PCR Cloning Kit (Agilent Technologies, Massy, France). The same procedure was carried out for the 617-bp *soxR* fragment, using primers SoxR F and SoxR R (Table 1). The empty plasmid pSC-A-amp/kan-E1, used as a negative control, was generated from pSC-A-amp/kan-*ramR* after digestion with EcoRI enzyme (Ozyme, Saint-Quentin-en-Yvelines, France) and ligation with the LigaFast Rapid DNA Ligation System (Promega, Charbonnières-les-Bains, France). All constructs were verified by sequencing them. The cloned wild-type *ramR* and *soxR* genes were transferred into electrocompetent *ramR* and *soxR* mutant strains, respectively, by transformation with the recombinant plasmids pSC-A-amp/kan-*ramR* and pSC-A-amp/kan-*soxR*.

Nucleotide sequence accession numbers. The sequences determined in this study have been submitted to GenBank, and their corresponding accession numbers are presented in Table 2.

RESULTS

Antibiotic resistance pattern of the mutants. Resistant mutants were obtained after one or two passages in low antibiotic concentrations from the susceptible KPBj Rev strains with a frequency of approximately 10^{-6} when ciprofloxacin or levofloxacin was used as a selector and 2×10^{-6} when cefoxitin was used. Four of the 9 mutants selected with cefoxitin presented a low level of resistance only to that molecule and remained susceptible to nalidixic acid and chloramphenicol. The remaining 5 mutants, similarly to the mutants selected with ciprofloxacin ($n = 6$) or levofloxacin ($n = 6$), displayed cross-resistance to the 3 antibiotics, suggesting the selection of mutants overexpressing an efflux system. These 17 mutants were retained for further analysis, after having been checked for the absence of mutations in both the *gyrA* and *parC* genes.

Transcription of the *acrB* gene. The relative transcription levels of *acrB* for the 3 KPBj Rev strains and their respective mutants are shown in Table 3. There was a notable increase in *acrB* transcription for all the mutants selected by both cefoxitin and fluoroquinolones from strains KPBj1 Rev and KPBj3 Rev compared with the parental strains: 2.9 to 6.1 times and 1.8 to 4.2 times, respectively. This increase (2.8 to 10 times) was observed for all of the mutants originating from strain KPBj5 Rev except one, mutant KPBj5 M1 Cip, for which the level of transcription of *acrB* was equivalent to that of the parental strain.

Sequences of the regulator genes. We analyzed the sequences of the regulator genes *acrR*, *ramR*, *ramA*, *marR*, *marA*, *soxR*, *soxS*, and *rob* of the 3 clinical isolates (KPBj E+), the 3 revertant strains (KPBj Rev), and the 17 mutants. First, we compared the sequences of the genes of each KPBj Rev strain with those of the 3 *K. pneumoniae* strains available in the GenBank database, i.e., *K. pneumoniae* subsp. *pneumoniae* MGH 78578 (accession number NC_009648) (25), *K. pneumoniae* 342 (accession number NC_011283) (11), and *K. pneumoniae* NTUH-K2044 (accession number NC_012731) (44). Whatever the gene, in strains KPBj1 Rev and KPBj5 Rev, the sequences were either strictly identical to those of strains MGH 78578 and NTUH-K2044 or displayed only one or two silent polymorphisms. For strain KPBj3 Rev, all the genes studied had sequences considerably different from those of strains MGH 78578 and NTUH-K2044 but very similar to those of strain 342, except for the *ramR* gene. Indeed, in strain 342, this gene showed a 96-bp deletion, resulting in the deletion of 32 amino acids approximately in the middle of the RamR protein. Such a deletion was not observed in the *ramR* gene of strain KPBj3 Rev, although the other polymorphisms characteristic of strain 342, compared with strain MGH 78578, were present.

Second, we compared the sequences of the regulator genes of the 3 clinical isolates and of the 17 mutants, which all showed cross-resistance to cefoxitin, nalidixic acid, and chloramphenicol, with those of the corresponding revertant strain. No mutation was detected in the 8 regulator genes studied from the 3 clinical isolates and 8 of the 17 mutants. However, point mutations within the *ramR* gene were detected in 8 of the 9 remaining mutants. In 7 of the *ramR* mutants, there were single-nucleotide substitutions, occurring at 6 different positions, whereas there was a deletion of 1 nucleotide in the last one. These genetic changes were distributed all along the *ramR*

TABLE 3. Relative transcription levels of the *acrB*, *ramA*, and *soxS* genes in the *in vitro*-selected mutants, their parental strains, and their wild-type *ramR* or *soxR* transformants (for *ramR* and *soxR* mutants, respectively) in comparison with strain ATCC 138821

Strain[a]	Gene transcription level (fold change)[b]		
	acrB	*ramA*	*soxS*
ATCC 138821	1	1	1
KPBj1 Rev	1.15 (±0.15)	1.05 (±0.44)	ND
ramR mutants			
KPBj1 M2 Cip	7.05 (±1.58)	31.62 (±16.67)	ND
KPBj1 M2 Cip T_0	5.49 (±3.87)	7.68 (±1.02)	ND
KPBj1 M2 Cip T_{ramR}	1.93 (±0.03)	0.19 (±0.07)	ND
KPBj1 M1 Cip	3.38 (±0.92)	43.91 (±30.32)	ND
KPBj1 M2 Lev	4.34 (±2.20)	38.10 (±9.24)	ND
KPBj1 M2 Lev T_{ramR}	1.27 (±0.25)	0.12 (±0.01)	ND
KPBj1 M3 Fox	5.81 (±2.41)	87.53 (±42.78)	ND
KPBj1 M3 Fox T_{ramR}	1.37 (±0.08)	0.14 (±0.04)	ND
Mutant with unknown mutation			
KPBj1 M1 Lev	4.01 (±0.40)	ND	ND
KPBj3 Rev	2.20 (±0.57)	2.31 (±2.92)	ND
ramR mutants			
KPBj3 M2 Fox	9.18 (±0.85)	37.50 (±18.97)	ND
KPBj3 M2 Fox T_{ramR}	1.52 (±0.36)	0.09 (±0.03)	ND
KPBj3 M3 Fox	6.48 (±1.32)	13.64 (±8.37)	ND
KPBj3 M3 Fox T_{ramR}	1.40 (±0.27)	0.06 (±0.001)	ND
Mutants with unknown mutations			
KPBj3 M1 Cip	4.04 (±0.40)	ND	ND
KPBj3 M2 Cip	4.94 (±0.07)	ND	ND
KPBj3 M1 Lev	4.54 (±0.26)	ND	ND
KPBj3 M2 Lev	5.50 (±0.06)	ND	ND
KPBj5 Rev	0.97 (±0.05)	2.59 (±0.34)	2.78 (±3.17)
ramR mutants			
KPBj5 M2 Lev	5.09 (±2.18)	84.21 (±72.24)	ND
KPBj5 M2 Lev T_{ramR}	1.45 (±0.34)	0.14 (±0.12)	ND
KPBj5 M3 Fox	3.33 (±0.82)	80.29 (±60.10)	ND
KPBj5 M3 Fox T_{ramR}	1.39 (±0.01)	0.10 (±0.06)	ND
soxR mutant			
KPBj5 M1 Cip	1.37 (±0.07)	3.38 (±0.97)	7.21 (±4.73)
KPBj5 M1 Cip T_{soxR}	1.66 (±0.11)	3.99 (±2.70)	2.34 (±0.33)
Mutants with unknown mutations			
KPBj5 M2 Cip	2.72 (±0.11)	ND	ND
KPBj5 M1 Lev	3.28 (±0.83)	ND	ND
KPBj5 M1 Fox	9.70 (±3.92)	ND	ND

[a] T_0, strain complemented with plasmid pSC-A-amp/kan-E1; T_{ramR}, strain complemented with plasmid pSC-A-amp/kan-*ramR* bearing the wild-type *ramR* gene; T_{soxR}, strain complemented with plasmid pSC-A-amp/kan-*soxR* bearing the wild-type *soxR* gene.

[b] ND, not determined. For gene expression levels, the results of two different experiments are indicated as mean (±standard deviation).

gene and led either to an amino acid exchange or to occurrence of a premature stop codon (directly or resulting from a frameshift mutation) and synthesis of a truncated RamR protein (Fig. 1). Finally, in the 9th mutant, KPBj5 M1 Cip, we found a point mutation in the *soxR* gene (C375G) that resulted in an amino acid substitution (N125K) in the SoxR protein (Fig. 2).

Functional impacts of the mutations observed in the *ramR* and *soxR* genes. The expression levels of the genes *ramA* and *soxS*, encoding the transcriptional activators RamA and SoxS, were measured for the mutants in which genetic changes were detected in *ramR* and *soxR*, respectively. In all the *ramR* mutants, we observed a dramatic increase in the *ramA* transcription level, up to 80 times that of the parental strain (Table 3). The *soxR* mutant, KPBj5 M1 Cip, showed an increase in the *soxS* transcription level compared to the parental strain, KPBj5 Rev, although this increase was lower than for the *ramA* gene (Table 3).

In order to demonstrate that the mutations found in the *ramR* and *soxR* regulator genes caused the observed efflux system overexpression, phenotypically characterized by antibiotic cross-resistance, we complemented the *ramR* and *soxR* mutants with the cloned wild-type *ramR* and *soxR* genes, respectively. For all the *ramR* mutants, introduction of the wild-type *ramR* gene strikingly lowered the transcription level of *ramA* and also suppressed the overexpression of the *acrB* gene (Table 3). For the *soxR* mutant, complementation with the wild-type *soxR* gene normalized the transcription level of *soxS*. However, it had no effect on *acrB* expression, consistent with the fact that KPBj5 M1 Cip was the only mutant that did not overexpress the *acrB* gene (Table 3).

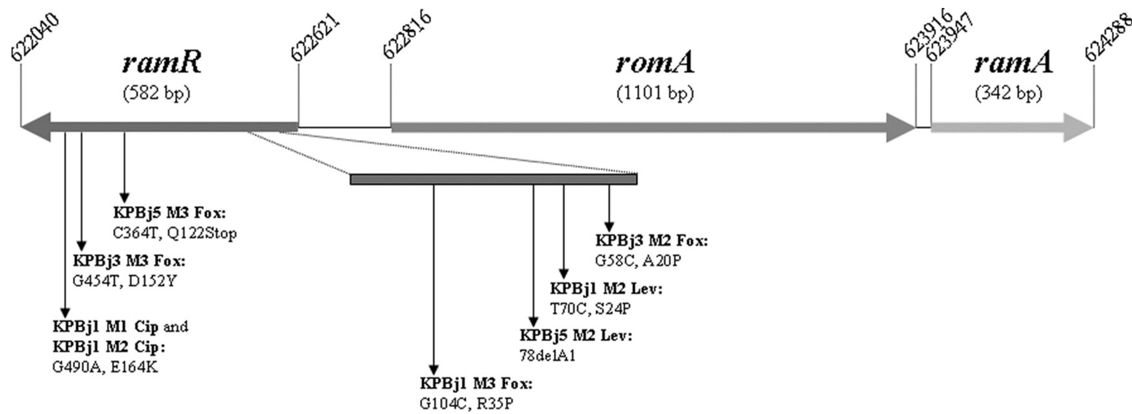

FIG. 1. Schematic diagram of the genomic region comprising the *ramR* and *ramA* genes in *K. pneumoniae* strain MGH 78578 (CP000647; accession number NC_009648). The mutations identified in the 7 *ramR* mutants and the deduced protein changes are clarified.

Impact of efflux system overexpression on antibiotic susceptibility. The MICs of members of different antibiotic families, including quinolones, chloramphenicol, cyclines, and β-lactams, among which were different penicillins, cephalosporins, and carbapenems, for the 3 clinical isolates, their 3 revertants, and the different types of mutants (with identified and with unknown mutations) that all displayed an overexpressed efflux-related resistance phenotype were measured (Table 4). When mutants selected with a given antibiotic from the same parental strain had the same sequence for their regulator genes (KPBj1 M1 Cip and KPBj1 M2 Cip, KPBj3 M1 Cip and KPBj3 M2 Cip, and KPBj3 M1 Lev and KPBj3 M2 Lev), MICs were determined for only one of them. As had previously been shown (3), the spontaneously reverted KPBj Rev strains obtained from the KPBj E+ clinical isolates were fully susceptible to all the antibiotics tested except amoxicillin and ticarcillin, consistent with the natural resistance profile of the *K. pneumoniae* species. For the 14 mutants tested, the MICs of quinolones were 4 to 32 times higher than for the parental strains. All of the mutants showed a significant increase in chloramphenicol (8 to 64 times), tetracycline (4 to 16 times), and tigecycline (4 to 16 times) MICs compared to the susceptible parental strains. β-Lactam MICs were also increased for the mutants, except for amoxicillin, alone or associated with clavulanic acid, imipenem, meropenem, and doripenem. This increase was always significant, whatever the mutant, for cefoxitin and cefuroxime (MICs 4 to 16 times higher than for the parental strains) and inconsistently significant for ticarcillin, cefazolin, cefotaxime, and ceftazidime (2 to 8 times) and for ertapenem (2 to 4 times). It is noteworthy that the MICs of all the antibiotics tested were similar for all the strains sharing an overexpressed efflux-related resistance phenotype and the *in vitro*-selected mutants, as well as the 3 clinical isolates.

Table 4 also shows that the transformation of the *ramR* and *soxR* mutants with the wild-type *ramR* and *soxR* genes, respectively, restored the strains' susceptibility to all the antibiotic families that are substrates of the efflux systems. The contribution of the wild-type regulator genes to susceptibility to amoxicillin, alone or associated with clavulanic acid, ticarcillin, and cefazolin, could not be evaluated because the pSC-A-amp/kan plasmid used for transformation harbored a *bla* gene responsible for high-level resistance to penicillins and first-generation cephalosporins.

FIG. 2. Schematic diagram of the genomic region comprising the *soxR* and *soxS* genes in *K. pneumoniae* strain MGH 78578 (CP000647; accession number NC_009648). The mutation identified in the *soxR* mutant and the deduced protein are clarified.

DISCUSSION

It has previously been shown in *K. pneumoniae* clinical isolates that efflux system overexpression results in cross-resistance to different families of antibiotics, including quinolones, chloramphenicol, tetracycline, tigecycline, and β-lactams (13, 21, 28, 29, 37). In this study, we showed the possibility of obtaining *in vitro* mutants displaying this cross-resistance as a result of antibiotic pressure exerted either with cefoxitin (the most impaired molecule within β-lactams) or with a fluoroquinolone (i.e., ciprofloxacin or levofloxacin). To obtain such mutants, we followed the procedure described by Miller et al. for the molecules with low endogenous resistance potential (22). However, it is noteworthy that, when cefoxitin was used, mutants with another kind of antibiotic resistance pattern could be selected: a low level of resistance to cefoxitin and full susceptibility to nalidixic acid and chloramphenicol. The mechanism explaining this single resistance to cefoxitin was not investigated in this study, but according to recently published data, porin alteration seems to be the most plausible hypothesis (3).

We had previously shown in *K. pneumoniae* clinical isolates that cefoxitin, quinolone, and chloramphenicol cross-resistance was not always associated with an increase in the transcription level of the genes encoding AcrAB, the most frequently investigated efflux pump in *Enterobacteriaceae* (3). Interestingly, such a feature was also found in one of our 17 mutants, KPBj5 M1

TABLE 4. Antibiotic susceptibilities of strain ATCC 138821, the 3 clinical isolates, their 3 revertants, the *in vitro*-selected mutants overexpressing an efflux system, and the *ramR* and *soxR* mutants complemented with the wild-type *ramR* and *soxR* genes, respectively

Strain	MIC (μg/ml)[a]																	
	NAL (≤8, >16)	CIP (≤0.5, >1)	LEV (≤1, >2)	CMP (≤8, >8)	TET (≤4, >8)	TGC (≤1, >2)	FOX (≤8, >32)	AMX (≤4, >8)	AMC (≤4/2, >8/2)	TIC (≤8, >16)	CF (≤8, >32)	CXM (≤8, >8)	CTX (≤1, >2)	CAZ (≤1, >8)	ETP (≤0.5, >1)	IMP (≤2, >8)	MEM (≤2, >8)	DOR (≤1, >4)
ATCC 138821	2	0.03	0.03	2	1	0.25	2	128	2	512	2	4	0.06	0.125	0.007	0.25	0.015	0.125
KPBj1 E+[b]	32	0.5	1	128	4	2	8	128	2	512	4	8	0.125	0.25	0.007	0.06	0.015	0.015
KPBj1 Rev	2	0.015	0.03	2	1	0.5	2	64	2	128	2	2	0.06	0.125	0.007	0.125	0.03	0.03
KPBj1 M2 Cip[c]	32	0.25	0.5	32	8	4	32	128	4	512	4	16	0.25	0.5	0.015	0.125	0.015	0.03
KPBj1 M2 Cip[c] T_0[e,h]	32	0.25	0.5	16	8	2	32	>1,024	>128	>1,024	16	16	0.25	0.5	0.03	0.125	0.015	0.03
KPBj1 M2 Cip T_{ramR}[f]	2	0.0075	0.03	1	0.5	0.25	2	>1,024	>128	>1,024	4	1	0.03	0.06	0.007	0.125	0.06	0.06
KPBj1 M2 Lev[c]	16	0.125	0.5	32	8	4	32	128	4	512	8	16	0.5	1	0.03	0.06	0.015	0.015
KPBj1 M2 Lev T_{ramR}	4	0.03	0.03	2	1	0.25	4	>1,024	>128	>1,024	8	2	0.06	0.125	0.015	0.125	0.06	0.06
KPBj1 M3 Fox[c]	16	0.125	0.5	32	16	4	32	128	4	512	8	16	0.5	1	0.03	0.06	0.015	0.015
KPBj1 M3 Fox T_{ramR}	2	0.015	0.03	1	0.5	0.25	2	>1,024	>128	>1,024	8	2	0.06	0.06	0.015	0.125	0.03	0.06
KPBj1 M1 Lev[b]	16	0.25	0.5	32	16	4	32	128	4	512	8	32	0.5	1	0.03	0.06	0.015	0.015
KPBj3 E+[b]	32	0.25	0.5	128	4	2	4	64	2	256	4	8	0.06	0.25	0.007	0.125	0.015	0.015
KPBj3 Rev	2	0.015	0.03	4	1	0.5	2	32	2	64	1	2	0.06	0.125	0.007	0.125	0.015	0.03
KPBj3 M2 Fox[c]	16	0.125	0.25	32	16	4	16	64	4	256	2	16	0.25	1	0.03	0.06	0.015	0.015
KPBj3 M2 Fox T_{ramR}	2	0.0075	0.03	2	1	0.25	2	>1,024	>128	>1,024	4	2	0.06	0.125	0.015	0.125	0.03	0.03
KPBj3 M3 Fox[c]	16	0.125	0.25	64	16	4	16	64	4	256	2	16	0.25	1	0.03	0.06	0.015	0.015
KPBj3 M3 Fox T_{ramR}	2	0.0075	0.03	2	1	0.25	2	>1,024	>128	>1,024	4	2	0.06	0.125	0.015	0.125	0.03	0.03
KPBj3 M1 Cip[b]	32	0.25	0.5	64	4	2	8	64	2	256	2	8	0.06	0.5	0.015	0.06	0.015	0.015
KPBj3 M1 Lev[b]	32	0.25	0.5	128	8	2	8	64	2	256	2	8	0.06	0.25	0.007	0.125	0.015	0.03
KPBj5 E+	8	0.06	0.125	8	4	2	8	256	2	1,024	4	8	0.125	0.25	0.015	0.125	0.03	0.06
KPBj5 Rev	2	0.015	0.06	2	1	0.5	2	128	2	256	2	2	0.06	0.125	0.007	0.125	0.03	0.06
KPBj5 M2 Lev[c]	16	0.125	0.5	32	16	4	32	256	4	1,024	8	16	0.5	1	0.03	0.06	0.015	0.03
KPBj5 M2 Lev T_{ramR}	2	0.03	0.03	2	1	0.25	2	>1,024	>128	>1,024	8	2	0.06	0.06	0.015	0.125	0.06	0.06
KPBj5 M3 Fox[c]	16	0.125	0.5	64	16	4	32	256	4	1,024	4	16	0.5	1	0.03	0.06	0.015	0.03
KPBj5 M3 Fox T_{ramR}	2	0.03	0.03	2	1	0.5	2	>1,024	>128	>1,024	8	2	0.06	0.06	0.015	0.125	0.06	0.06
KPBj5 M1 Cip[d]	16	0.125	0.25	16	8	2	16	256	2	512	4	8	0.125	0.5	0.015	0.06	0.015	0.03
KPBj5 M1 Cip T_{soxR}[g]	2	0.03	0.03	2	1	0.25	2	>1,024	>128	>1,024	8	2	0.06	0.06	0.015	0.125	0.03	0.03
KPBj5 M2 Cip[b]	64	0.5	1	128	8	2	16	256	2	1,024	4	8	0.125	0.5	0.015	0.06	0.015	0.03
KPBj5 M1 Lev[b]	64	0.5	1	128	8	2	16	256	2	1,024	4	8	0.125	0.25	0.015	0.125	0.015	0.03
KPBj5 M1 Fox[b]	64	0.125	0.25	128	16	4	32	128	2	512	4	16	0.25	0.5	0.03	0.03	0.015	0.007

[a] NAL, nalidixic acid; CIP, ciprofloxacin; LEV, levofloxacin; CMP, chloramphenicol; TET, tetracycline; TGC, tigecycline; FOX, cefoxitin; AMX, amoxicillin; AMC, amoxicillin associated with clavulanic acid; TIC, ticarcillin; CF, cefazolin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; ETP, ertapenem; IMP, imipenem; MEM, meropenem; DOR, doripenem. The breakpoints according to the French Antibiogram Committee are given in parentheses.

[b] Overexpression of *acrB*.

[c] Overexpression of gees *acrB* and *ramA*.

[d] Overexpression of *soxR* and *soxS*.

[e] T_0, strain complemented with plasmid pSC-A-amp/kan-E1.

[f] T_{ramR}, strain complemented with plasmid pSC-A-amp/kan-*ramR* bearing the wild-type *ramR* gene.

[g] T_{soxR}, strain complemented with plasmid pSC-A-amp/kan-*soxR* bearing the wild-type *soxR* gene.

[h] For the strains bearing plasmid pSC-A-amp/kan with or without a cloned gene, the MICs of AMX, AMC, TIC, and CEF are greatly increased because the plasmid harbors a penicillinase-encoding gene.

Cip. The analysis of the sequences of the genes regulating efflux pump expression revealed that the *soxR* gene was mutated in this strain, leading to *soxS* overexpression. We demonstrated the involvement of this genetic modification in the expression of a multidrug-resistant efflux phenotype through the complementation of the mutant with the wild-type *soxR* gene. The *soxR* mutation observed led to the replacement of an asparagine residue by a lysine residue at position 125, which is located close to the C-terminal cluster of 4 cysteine residues (Cys110, Cys113, Cys115, and Cys121) that has been demonstrated to be essential for the binding of the [2Fe-2S] centers and for the activity of SoxR in *E. coli* (4). The fact that the SoxR protein is a transcriptional activator of *soxS* and not a repressor may explain why a single *soxR* mutant (versus 8 *ramR* mutants) was obtained among the 17 mutants studied. Indeed, a mutation leading to constitutive activation of a regulator protein is probably more difficult to achieve that an inactivating one. A point mutation in this region of the *soxR* gene has already been described in a multidrug-resistant clinical isolate of *S. enterica*, but whether this mutation influenced the expression of AcrAB or another efflux pump was not explored (19). To our knowledge, this is the first time that the involvement of the *soxR* gene in the regulatory pathways controlling the expression of an efflux pump in *K. pneumoniae* has been shown. However, a mutated *soxR* gene was not observed in the *K. pneumoniae* clinical isolate in which the transcription level of the *acrB* gene was not increased (3). Overall, these results strongly suggest the existence of an efflux pump(s) other than AcrAB in *K. pneumoniae*.

As previously reported in 5 clinical isolates of *K. pneumoniae* with reduced susceptibility to tigecycline (14), we found point mutations in the *ramR* genes of 8 of our 17 mutants, and these mutations were shown to be responsible for overexpression of both the *ramA* and *acrB* genes. However, their locations in the gene sequence were different from those described by Hentschke et al. (14). Still, by complementation experiments, we demonstrated that RamR, the local repressor of *ramA*, is really involved in the regulation of AcrAB expression in *K. pneumoniae*, as has been demonstrated in *S. enterica* (1). Whether RamA controls AcrAB expression directly or indirectly through interplay with other global regulators, as previously suggested by some authors (5, 8, 27), was not studied here.

In vitro ramR mutants were obtained at a high frequency, irrespective of the selector used, i.e., cefoxitin or fluoroquinolones. Such a result might suggest that *ramR* mutants could easily be obtained *in vivo*. Nevertheless, we did not observe *ramR* mutants among the 3 clinical isolates that we analyzed, as we did not observe *soxR* mutants. The most surprising observation was the absence of genetic modification in the 8 regulator genes studied among the 3 clinical isolates. Alignment of the sequences of their regulator genes with those available in the GenBank database revealed that 2 of our clinical isolates were very similar to the 2 published *K. pneumoniae* strains of clinical origin: MGH 78578, isolated from a patient with pneumonia (25), and NTUH-K2044, isolated from a patient with liver abscess and meningitis (44). On the other hand, our third clinical isolate strongly resembled *K. pneumoniae* 342, a nitrogen-fixing endophyte strain isolated from the interior of maize plants (11), except for the 96-bp deletion in the *ramR* gene. Also, we did not observe any mutation in 8 of the 17 mutants studied, although all showed a typical overexpressed efflux-related antibiotic resistance pattern, like the 3 clinical isolates, and all had an increased *acrB* transcription level, like 2 clinical isolates. This finding highlights the complexity of the regulatory pathways that are involved in the control of AcrAB expression in *K. pneumoniae*. We hypothesize that mutations in other regulator regions, first in the binding sites of the transcriptional activators and/or repressors, could explain the overexpression of AcrAB and possibly of other efflux pumps not yet identified.

Finally, this study allowed us to specify the β-lactam antibiotics whose activities are impaired by efflux system overexpression. Considering our results, it seems clear that, besides cefoxitin, first-generation (cefazolin), second-generation (cefuroxime), and even third-generation (cefotaxime and ceftazidime) cephalosporins are substrates of efflux pumps. Our findings seem to contradict a study by Källman et al. (17) in which the authors concluded that the nonsusceptibility to cefuroxime observed in multidrug-resistant *K. pneumoniae* isolates was not related to efflux. They based this conclusion on the absence of cefuroxime activity restoration when the efflux pump inhibitor phenylalanine arginine β-naphthylamide (PAβN) was used. However, this argument appears inadequate, since our team showed recently that the use of both cloxacillin and PAβN was required to inhibit the efflux of β-lactams (29). Regarding carbapenems, MICs of imipenem, meropenem, and doripenem were not significantly increased in both clinical isolates and the different types of mutants, whereas ertapenem MICs were significantly increased in some mutants, notably in the *ramR* mutants.

In conclusion, beyond suggesting the complex regulation of efflux system expression in *K. pneumoniae*, this study demonstrated that a large panel of antibiotics, including those widely used for a long time for treating human infectious diseases (fluoroquinolones and various β-lactams) and those more recently commercialized (tigecycline and ertapenem), are substrates of efflux systems in this species. Although we showed that mutants overexpressing efflux systems display a low level of resistance to the majority of the antibiotics studied, it is reasonable to think that this is a threatening mechanism of resistance. Indeed, its association with other mechanisms of resistance, notably to β-lactams (β-lactamase production and/or porin alteration), could provide advantages for bacterial survival, as suggested by our previous study on *K. pneumoniae* virulence in the *Caenorhabditis elegans* model and by the cefoxitin resistance that was present in the first ESBL-producing *K. pneumoniae* strain responsible for outbreaks in France (3, 16).

REFERENCES

1. **Abouzeed, Y. M., S. Baucheron, and A. Cloeckaert.** 2008. *ramR* mutations involved in efflux-mediated multidrug resistance in *Salmonella enterica* serovar Typhimurium. Antimicrob. Agents Chemother. **52:**2428–2434.
2. **Alekshun, M. N., and S. B. Levy.** 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. Antimicrob. Agents Chemother. **41:**2067–2075.
3. **Bialek, S., et al.** 2010. Membrane efflux and influx modulate both multidrug resistance and virulence of *Klebsiella pneumoniae* in a *Caenorhabditis elegans* model. Antimicrob. Agents Chemother. **54:**4373–4378.
4. **Bradley, T. M., E. Hidalgo, V. Leautaud, H. Ding, and B. Demple.** 1997. Cysteine-to-alanine replacements in the *Escherichia coli* SoxR protein and the role of the [2Fe-2S] centers in transcriptional activation. Nucleic Acids Res. **25:**1469–1475.

5. **Bratu, S., D. Landman, A. George, J. Salvani, and J. Quale.** 2009. Correlation of the expression of *acrB* and the regulatory genes *marA*, *soxS* and *ramA* with antimicrobial resistance in clinical isolates of *Klebsiella pneumoniae* endemic to New York City. J. Antimicrob. Chemother. **64:**278–283.
6. **Buckley, A. M., et al.** 2006. The AcrAB-TolC efflux system of *Salmonella enterica* serovar Typhimurium plays a role in pathogenesis. Cell. Microbiol. **8:**847–856.
7. **Chen, J. Y., et al.** 2001. Molecular epidemiology and mutations at *gyrA* and *parC* genes of ciprofloxacin-resistant *Escherichia coli* isolates from a Taiwan medical center. Microb. Drug Resist. **7:**47–53.
8. **Chollet, R., J. Chevalier, C. Bollet, J. M. Pages, and A. Davin-Regli.** 2004. RamA is an alternate activator of the multidrug resistance cascade in *Enterobacter aerogenes*. Antimicrob. Agents Chemother. **48:**2518–2523.
9. **Cohen, S. P., H. Hachler, and S. B. Levy.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175:**1484–1492.
10. **Doumith, M., M. J. Ellington, D. M. Livermore, and N. Woodford.** 2009. Molecular mechanisms disrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK. J. Antimicrob. Chemother. **63:**659–667.
11. **Fouts, D. E., et al.** 2008. Complete genome sequence of the N_2-fixing broad host range endophyte *Klebsiella pneumoniae* 342 and virulence predictions verified in mice. PLoS Genet. **4:**e1000141.
12. **George, A. M., R. M. Hall, and H. W. Stokes.** 1995. Multidrug resistance in *Klebsiella pneumoniae*: a novel gene, *ramA*, confers a multidrug resistance phenotype in *Escherichia coli*. Microbiology **141:**1909–1920.
13. **Hasdemir, U. O., J. Chevalier, P. Nordmann, and J. M. Pages.** 2004. Detection and prevalence of active drug efflux mechanism in various multidrug-resistant *Klebsiella pneumoniae* strains from Turkey. J. Clin. Microbiol. **42:** 2701–2706.
14. **Hentschke, M., M. Wolters, I. Sobottka, H. Rohde, and M. Aepfelbacher.** 2010. *ramR* mutations in clinical isolates of *Klebsiella pneumoniae* with reduced susceptibility to tigecycline. Antimicrob. Agents Chemother. **54:**2720–2723.
15. **Imuta, N., et al.** 2008. The *Escherichia coli* efflux pump TolC promotes aggregation of enteroaggregative *E. coli* 042. Infect. Immun. **76:**1247–1256.
16. **Jarlier, V., M. H. Nicolas, G. Fournier, and A. Philippon.** 1988. Extended broad-spectrum β-lactamases conferring transferable resistance to newer ß-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. **10:**867–878.
17. **Källman, O., et al.** 2008. Cefuroxime non-susceptibility in multidrug-resistant *Klebsiella pneumoniae* overexpressing *ramA* and *acrA* and expressing *ompK35* at reduced levels. J. Antimicrob. Chemother. **62:**986–990.
18. **Keynan, Y., and E. Rubinstein.** 2007. The changing face of *Klebsiella pneumoniae* infections in the community. Int. J. Antimicrob. Agents **30:**385–389.
19. **Koutsolioutsou, A., E. A. Martins, D. G. White, S. B. Levy, and B. Demple.** 2001. A *soxRS*-constitutive mutation contributing to antibiotic resistance in a clinical isolate of *Salmonella enterica* (serovar Typhimurium). Antimicrob. Agents Chemother. **45:**38–43.
20. **Martinez-Martinez, L.** 2008. Extended-spectrum beta-lactamases and the permeability barrier. Clin. Microbiol. Infect. **14**(Suppl. 1)**:**82–89.
21. **Mazzariol, A., J. Zuliani, G. Cornaglia, G. M. Rossolini, and R. Fontana.** 2002. AcrAB efflux system: expression and contribution to fluoroquinolone resistance in *Klebsiella* spp. Antimicrob. Agents Chemother. **46:**3984–3986.
22. **Miller, K., A. J. O'Neill, and I. Chopra.** 2002. Response of *Escherichia coli* hypermutators to selection pressure with antimicrobial agents from different classes. J. Antimicrob. Chemother. **49:**925–934.
23. **Nikaido, H., and Y. Takatsuka.** 2009. Mechanisms of RND multidrug efflux pumps. Biochim. Biophys. Acta **1794:**769–781.
24. **Oethinger, M., I. Podglajen, W. V. Kern, and S. B. Levy.** 1998. Overexpression of the *marA* or *soxS* regulatory gene in clinical topoisomerase mutants of *Escherichia coli*. Antimicrob. Agents Chemother. **42:**2089–2094.
25. **Ogawa, W., et al.** 2005. Multidrug resistance in *Klebsiella pneumoniae* MGH78578 and cloning of genes responsible for the resistance. Biol. Pharm. Bull. **28:**1505–1508.
26. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major rôle in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178:**306–308.
27. **O'Regan, E., et al.** 2009. Multiple regulatory pathways associated with high-level ciprofloxacin and multidrug resistance in *Salmonella enterica* serovar enteritidis: involvement of RamA and other global regulators. Antimicrob. Agents Chemother. **53:**1080–1087.
28. **Padilla, E., et al.** 2010. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. Antimicrob. Agents Chemother. **54:**177–183.
29. **Pages, J. M., et al.** 2009. Efflux pump, the masked side of β-lactam resistance in *Klebsiella pneumoniae* clinical isolates. PLoS One **4:**e4817.
30. **Papanicolaou, G. A., A. A. Medeiros, and G. A. Jacoby.** 1990. Novel plasmid-mediated β-lactamase (MIR-1) conferring resistance to oxyimino- and a-methoxy β-lactams in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **34:**2200–2209.
31. **Pérez, A., et al.** 2007. Cloning, nucleotide sequencing, and analysis of the AcrAB-TolC efflux pump of *Enterobacter cloacae* and determination of its involvement in antibiotic resistance in a clinical isolate. Antimicrob. Agents Chemother. **51:**3247–3253.
32. **Piddock, L. J.** 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin. Microbiol. Rev. **19:**382–402.
33. **Piddock, L. J., D. G. White, K. Gensberg, L. Pumbwe, and D. J. Griggs.** 2000. Evidence for an efflux pump mediating multiple antibiotic resistance in *Salmonella enterica* serovar Typhimurium. Antimicrob. Agents Chemother. **44:**3118–3121.
34. **Podschun, R., and U. Ullmann.** 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. **11:**589–603.
35. **Pradel, E., and J. M. Pages.** 2002. The AcrAB-TolC efflux pump contributes to multidrug resistance in the nosocomial pathogen *Enterobacter aerogenes*. Antimicrob. Agents Chemother. **46:**2640–2643.
36. **Ruzin, A., D. Keeney, and P. A. Bradford.** 2005. AcrAB efflux pump plays a role in decreased susceptibility to tigecycline in *Morganella morganii*. Antimicrob. Agents Chemother. **49:**791–793.
37. **Ruzin, A., M. A. Visalli, D. Keeney, and P. A. Bradford.** 2005. Influence of transcriptional activator RamA on expression of multidrug efflux pump AcrAB and tigecycline susceptibility in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **49:**1017–1022.
38. **Schneiders, T., S. G. Amyes, and S. B. Levy.** 2003. Role of AcrR and RamA in fluoroquinolone resistance in clinical *Klebsiella pneumoniae* isolates from Singapore. Antimicrob. Agents Chemother. **47:**2831–2837.
39. **Sirot, D., R. Labia, A. Morand, and P. Courvalin.** 1987. Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel β-lactamase. J. Antimicrob. Chemother. **20:**323–334.
40. **Sulavik, M. C., M. Dazer, and P. F. Miller.** 1997. The *Salmonella typhimurium mar* locus: molecular and genetic analyses and assessment of its role in virulence. J. Bacteriol. **179:**1857–1866.
41. **Visalli, M. A., E. Murphy, S. J. Projan, and P. A. Bradford.** 2003. AcrAB multidrug efflux pump is associated with reduced levels of susceptibility to tigecycline (GAR-936) in *Proteus mirabilis*. Antimicrob. Agents Chemother. **47:**665–669.
42. **Webber, M. A., A. Talukder, and L. J. Piddock.** 2005. Contribution of mutation at amino acid 45 of AcrR to *acrB* expression and ciprofloxacin resistance in clinical and veterinary *Escherichia coli* isolates. Antimicrob. Agents Chemother. **49:**4390–4392.
43. **Wu, J., W. R. Dunham, and B. Weiss.** 1995. Overproduction and physical characterization of SoxR, a [2Fe-2S] protein that governs an oxidative response regulon in *Escherichia coli*. J. Biol. Chem. **270:**10323–10327.
44. **Wu, K. M., et al.** 2009. Genome sequencing and comparative analysis of *Klebsiella pneumoniae* NTUH-K2044, a strain causing liver abscess and meningitis. J. Bacteriol. **191:**4492–4501.
45. **Yigit, H. Q., et al.** 2001. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **45:**1151–1161.