Complete Deletion of the *ramR* Gene in an *In Vitro*-Selected Mutant of *Klebsiella pneumoniae* Overexpressing the AcrAB Efflux Pump

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In *Klebsiella pneumoniae*, overexpression of nonspecific efflux pumps causes low-level cross-resistance to β -lactams (mainly cefoxitin) and to antibiotics belonging to other families, such as quinolones, chloramphenicol, and tetracycline (1, 2). Among these efflux systems, the pump AcrAB seems to be the one most involved in antibiotic resistance in *Enterobacteriaceae*, including *K. pneumoniae* (3–8).

Expression of the *acrAB* operon is controlled by (i) its local repressor AcrR (9) and (ii) the global transcriptional activators MarA, SoxS, Rob, and, except for *Escherichia coli*, RamA (10, 11). Mutations in the *ramR* gene, coding the recently identified local repressor of *ramA*, in multidrug-resistant clinical isolates of *Salmonella enterica* (12) and, more recently, *K. pneumoniae* (13) have been reported. In a previous work, we also found point mutations in *ramR* in eight mutants of *K. pneumoniae* selected *in vitro* with cefoxitin or fluoroquinolones (14). Here, we report for the first time the deletion of the entire *ramR* gene in a laboratory-derived mutant of *K. pneumoniae* overexpressing the AcrAB efflux pump.

This mutant, called KPBj1 M3 Lev, was obtained from KPBj1 Rev (15), according to the procedure described previously (14). Briefly, an aliquot of an overnight culture of the parental strain in Mueller-Hinton (MH) broth containing levofloxacin at a concentration of 0.25 $\times$ the MIC (0.008 mg/liter) was plated on MH agar containing 4 $\times$ the original MIC (0.128 mg/liter).

Susceptibility of the two strains to antibiotics was determined by the agar dilution method. Whereas KPBj1 Rev was susceptible to nalidixic acid, chloramphenicol, tetracycline, and cefoxitin, KPBj1 M3 Lev displayed cross-resistance to the four antibiotics (Table 1).

Real-time reverse transcription-PCR experiments showed enhanced expression of both the *acrB* and *ramA* genes for the mutant, in comparison with the parental strain (Table 1).

PCR designed for amplification of the *ramR* gene (14) yielded, for KPBj1 M3 Lev, a DNA fragment of about 175 bp, instead of the

expected 895-bp fragment. Sequencing of this DNA fragment revealed a deletion of 720 bp encompassing the entire *ramR* gene (Fig. 1).

Finally, complementation of the mutant with the wild-type *ramR* gene was performed as described previously (14). The transcription level of gene *acrB* was normalized in the transformed strain KPBj1 M3 Lev *T_{ramR}* and that of gene *ramA* was considerably lowered, even compared to the parental strain. Introduction of the wild-type *ramR* gene restored the strain's susceptibility to antibiotics, whereas transformation with the control plasmid had no significant effect (Table 1). These results clearly show that the *ramR* deletion reported here is sufficient to explain the phenotype observed for our mutant.

Homologous recombination between two identical or closely related regions present in the same orientation in a single DNA molecule can lead to the excision of the fragment bordered by the repeats (16). As indicated in Fig. 1, such a genetic event, involving the directly repeated "agtngacgtg" sequences found on both sides of the deleted portion, occurred in our mutant. This mechanism has already been observed in *E. coli* with regard to the loss of the *ampR* gene, which encodes the activator of *ampC* in *Enterobacter cloacae* (17).

In silico comparison of the flanking regions of *ramR*, in several *Enterobacteriaceae* strains, showed that the *K. pneumoniae* direct repeats cited above are absent in *S. enterica* serovar Typhimurium LT2, *E. cloacae* EcWSU1, and *E. aerogenes* KCTC

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TABLE 1 Antibiotic susceptibility and relative transcription levels of the *acrB* and *ramA* genes of the *in vitro*-selected mutant, its parental strain, and its wild-type *ramR* transformant

Strain ^a	MIC (μ g/ml) ^b				Gene transcription level (fold change $\pm$ SD) ^c	
	NAL (≤ 8 , > 16)	CMP (≤ 8 , > 8)	TET (≤ 4 , > 8)	FOX (≤ 8 , > 32)	<i>acrB</i>	<i>ramA</i>
ATCC 13883	ND	ND	ND	ND	1	1
KPBj1 Rev	2	2	1	2	1.15 (± 0.15)	1.05 (± 0.44)
KPBj1 M3 Lev	16	32	16	32	3.74 (± 0.37)	22.46 (± 18.56)
KPBj1 M3 Lev <i>T_{ramR}</i>	4	4	1	2	1.34 (± 0.14)	0.37 (± 0.01)
KPBj1 M3 Lev <i>T₀</i>	16	32	16	32	4.94 (± 1.48)	14.94 (± 10.21)

^a *T_{ramR}* strain complemented with plasmid pSC-A-amp/kan-*ramR* bearing the wild-type *ramR* gene; *T₀* strain complemented with plasmid pSC-A-amp/kan-E1.

^b NAL, nalidixic acid; CMP, chloramphenicol; TET, tetracycline; FOX, cefoxitin; ND, not determined. The breakpoints according to the French Antibiogram Committee are given in parentheses.

^c The results of two different experiments are indicated as means ($\pm$ standard deviations).

FIG 1 Aligned sequences of the flanking regions of the *ramR* gene in *Klebsiella pneumoniae* MGH 78578 (GenBank accession number NC_009648; positions 621908 to 622769), *Enterobacter aerogenes* KCTC2190 (accession number NC_005663; positions 2894997 to 2895853), *Salmonella enterica* serovar Typhimurium LT2 (accession number NC_003197; positions 638014 to 638868), and *E. cloacae* EcWSU1 (accession number NC_0016514; positions 1121395 to 1122250). The direct repeats involved in the deletion of *ramR* in the mutant KPBj1 M3 Lev are indicated in bold characters in the sequence of *K. pneumoniae*, and the deleted portion is boxed. Potential sites of homologous recombination identified in the three other strains are underlined. Nucleotide positions conserved in the four strains are marked with an asterisk.

2190. However, we detected in those three strains some other potential sites of homologous recombination present on both sides of *ramR* (Fig. 1).

Taking these findings together, this is the first time, to our knowledge, that a complete deletion of the *ramR* gene has been found in an *Enterobacteriaceae* strain. The fact that our mutant was selected after a single step of culture in a medium containing low concentrations of levofloxacin suggests, however, that *ramR* deletion is quite easy to achieve and that it could occur in clinical isolates selected under antibiotic pressure.

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