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Detection and Prevalence of Active Drug Efflux Mechanism in Various Multidrug-Resistant *Klebsiella pneumoniae* Strains from Turkey

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The prevalence of active drug efflux pump and porin alterations was investigated in Turkish nosocomial strains of *Klebsiella pneumoniae* exhibiting a multidrug-resistant phenotype. MICs of various antibiotics, including quinolones, chloramphenicol, tetracycline, and β-lactams, for those strains were determined either with or without the efflux pump inhibitor phenylalanine arginine β-naphthylamide (PAβN). Thirty-nine percent of the strains exhibited a PAβN-modulated resistance for quinolones, chloramphenicol, and tetracycline. In these strains, a significant increase of chloramphenicol accumulation was gained in the presence of the efflux pump inhibitor PAβN or with the energy uncoupler carbonyl cyanide *m*-chlorophenylhydrazone. Moreover, high-level expression of the membrane fusion protein AcrA, which was immunodetected in most of those isolates, suggests that the AcrAB/TolC efflux machinery contributed to their antibiotic resistance. Studies of *K. pneumoniae* porins indicated that the majority of the strains, including extended-spectrum β-lactamase producers and efflux-positive ones, presented an alteration in their sorbitol-sensitive porin (OmpK35) expression. This is the first report showing the prominent role of active drug efflux in the antibiotic resistance of nosocomial *K. pneumoniae* strains from Turkey.

Increasing prevalence of multiple-antibiotic resistance among nosocomial strains of gram-negative bacteria is an emerging problem worldwide. The role of target modifications and enzymatic modifications of the drugs in the multiple antibiotic resistance of bacteria has been extensively reported, whereas more rare reports underlined the participation of multidrug efflux pump systems in the multidrug-resistant (MDR) phenotype (1, 5, 12, 17, 20–22, 26). Genes encoding MDR pumps are normal constituents of bacterial chromosomes and thus provide to bacteria the intrinsic potential to develop the MDR phenotype without acquisition of antibiotic resistance genes (5, 6). The activation of multidrug efflux pump genes by mutations or induction caused by stress of exposure to xenobiotics results in overexpression of pumps (1, 5, 6, 22, 24). Thus, bacteria may become resistant to most of the antibiotics that are expelled by these efflux machineries. Among MDR efflux pump mechanisms, AcrAB/TolC in *Escherichia coli* and several Mex pumps in *Pseudomonas aeruginosa* have been well studied, such as the resistance-nodulation-division active drug efflux systems (5, 17, 20–22, 24). Similar resistance-nodulation-division pumps are involved in other gram-negative bacterial species, including *Klebsiella* spp., *Enterobacter* spp., and *Salmonella* spp. (5, 16, 18, 26, 27). It is also important to note that in several documented MDR clinical isolates, the efflux mechanism is often associated with a modification of outer membrane permeability via the loss of major porins (5, 21, 22, 25).

The main goal of the present study was to determine the role of the phenotype of drug efflux in several MDR *Klebsiella pneumoniae* clinical isolates.

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MATERIALS AND METHODS

Bacterial strains and media. Eighteen MDR *K. pneumoniae* strains were isolated from different patients during 6 months in 2002 at the University Hospital in Istanbul. The origins of the strains are shown in Tables 1 and 2. Identification of strains was performed by using both the VITEK automated system (bioMérieux, Marcy l'Etoile, France) and API 20E (Api-bioMérieux Systems). *K. pneumoniae* ATCC 11296 was used as the reference strain in determinations of MICs for the various clinical isolates. *K. pneumoniae* Kp63, a porin-deficient clinical strain (2); *K. pneumoniae* ATCC 11296; *E. coli* BW5104 (26), which expresses AcrA at a basal level; and *Enterobacter aerogenes* ATCC 13048(pJS04) (*ompX* mutant with its promoter [29]), a strain that overexpresses OmpX, were used as controls for protein analysis. Bacteria were grown either in Mueller-Hinton (MH) broth and agar or in Luria-Bertani (LB) broth or nutrient broth (Difco Laboratories, Detroit, Mich.) at 37°C.

Antibiotic susceptibility testing and ESBL detection. MIC determinations for four structurally unrelated classes of bacterial drugs were carried out using a twofold broth dilution method in MH broth according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (19) to evaluate the MDR phenotype. The following antibiotics were purchased from Sigma Chemical Co. (Saint Quentin Fallavier, France): ofloxacin, norfloxacin, nalidixic acid, chloramphenicol, and tetracycline. Cefepime, ceftazidime, cefoxitin, meropenem, and imipenem were provided by Bristol-Myers Squibb (Syracuse, N.Y.), Roche (Neuilly-Sur-Seine, France), Glaxo-Wellcome S.p.A (Verona, Italy), Pan-Pharma S.A. (Fougères, France), Imperial Chemical Industries PLC (London, Great Britain), Merck Sharp and Dohme (Chibert, France), and Sanofi-Synthelabo (Paris, France), respectively. MIC determinations were also carried out with fixed concentrations (50 μM) of the efflux pump inhibitor phenylalanine arginine β-naphthylamide (PAβN) (Sigma Chemical Co.) against quinolones, chloramphenicol, tetracycline, and cefepime (4, 13). The double-disk synergy test was used as a screening test for detecting extended-spectrum β-lactamase (ESBL)-producing strains. Cefotaxime (30-μg), ceftazidime (30-μg), and aztreonam (30-μg) disks were placed on MH agar adjacent to a clavulanate-amoxicillin disk (20 μg of amoxicillin plus 10 μg of clavulanate). Antibiotic-containing disks were from Becton Dickinson Microbiology Systems (Sparks, Md.). The procedures and interpretation of the double-disk synergy test were as described previously (10).

Chloramphenicol accumulation test. Measurement of [^{14}C]chloramphenicol uptake by intact cells was adapted from methods described in previous studies (2, 4). Exponential-phase bacteria grown in LB broth were pelleted, washed once, and suspended to a density of 10^{10} CFU/ml in 50 mM sodium phosphate buffer,

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TABLE 1. Susceptibilities of *K. pneumoniae* isolates in group A to various antibiotics with and without an efflux pump inhibitor, PAβN

Strain	Source[d] of strain	MIC (μg/ml)[a]										
		OFX	NOR	NAL	CHL	TET	FEP	CAZ	FOX	IPM	MEM	ESBL
K80	TA[d]	32	32	≥512	128	≥128	2	8	16	0.125	≤0.06	−
K80 + PAβN[b]		1	8	32	2	≥128	1					
K89	Blood	32	256	≥512	256	≥128	2	1	32	0.5	≤0.06	−
K89 + PAβN		0.5	4	32	2	≥128	0.125					
K2	Urine	2	2	64	32	8	16	≥512	64	1	0.25	+
K2 + PAβN		≤0.06	0.25	0.25	1	0.25	4					
K32	Wound	0.125	0.25	16	16	8	32	≥512	64	1	≤0.06	+
K32 + PAβN		≤0.06	0.125	0.25	1	2	8					
K33	Urine	1	1	32	≥512	≥128	2	64	8	0.25	≤0.06	+
K33 + PAβN		≤0.06	0.125	0.25	32	≥128	1					
K74	TA	1	1	16	32	8	16	≥512	64	1	≤0.06	+
K74 + PAβN		≤0.06	0.25	0.25	0.5	4	4					
K121	CSF	0.125	0.5	4	64	4	128	≥512	64	1	0.125	+
K121 + PAβN		≤0.06	0.125	0.25	0.5	0.125	ND[e]					
ATCC 11296[c]		0.125	0.25	8	8	1	0.125	0.5	4	0.5	ND	ND

[a] Antibiotics were tested alone or with PAβN. MICs were determined in MH broth according to NCCLS guidelines (19). MICs were obtained from three independent measurements. Abbreviations: OFX, ofloxacin; NOR, norfloxacin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; FEP, cefepime; CAZ, ceftazidine; FOX cefoxitin; IPM, imipenem; MEM, meropenem.
[b] PAβN, 50 μM PAβN in each test tube.
[c] *K. pneumoniae* reference strain.
[d] Abbreviations: TA, tracheal aspiration; CSF, cerebrospinal fluid. ESBL, extended-spectrum β-lactamase.
[e] ND, not determined.

pH 7, containing 5 mM magnesium chloride. [^{14}C]chloramphenicol (specific radioactivity, 59.46 mCi/mmol) was added to 600 μl of cell suspension at 37°C in a shaking water bath, yielding a final chloramphenicol concentration of 5 μM. At various intervals, 100 μl of the suspension was removed and immediately filtered through GF/C filters (Whatman, Maidstone, Kent, United Kingdom). After three washes with 5 ml of 50 mM sodium phosphate buffer (pH 7) containing 0.1 M lithium chloride, the filters were dried and the radioactivity was measured by using a Packard scintillation counter. Inhibition assays were performed in the presence of an energy uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and PAβN at final inhibitor concentrations of 50 and 200 μM, respectively. The MICs of CCCP were above 500 μM for strains ATCC 11296, K80, K89, and K128 (data not shown).

SDS-polyacrylamide gel electrophoresis and immunodetection of AcrA, OmpA, OmpX, and porins. Cell pellets were prepared from exponential-phase bacteria grown in MH broth and then they were solubilized in loading buffer at 96°C. Samples (0.02 optical density at 600 nm) were loaded on sodium dodecyl sulfate (SDS)-polyacrylamide gels (10% polyacrylamide, 0.1% SDS) and run at 160 V for 1 h (2). Electrotransfer of the resulting bands to nitrocellulose membranes was carried out with 0.05% SDS. After an initial saturating step with Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 8) containing 10% skim milk powder at 4°C, nitrocellulose membranes were incubated in Tris-buffered saline containing 10% bovine serum and 0.2% Triton X-100 for 2 h at room temperature in the presence of polyclonal antibodies directed against AcrA or OmpA protein (14, 26). After three washes with the same buffer, detection was performed with alkaline phosphatase-conjugated affinitiPure goat anti-rabbit immunoglobulin G antibodies (Jackson Immuno-Research, West Grove, Pa.). Evaluation of the AcrA and OmpA amounts was made by measuring the intensity of immunoblotting bands exhibited by clinical and control strains. The level of AcrA expression in the control strains (*E. coli* BW5104 and *K. pneumoniae* ATCC 11296) was rated as positive (+), and that in the strains overexpressing AcrA protein was recorded as slightly or highly overexpressed (++ and +++, respectively). For OmpX, the same conditions were applied as described for OmpA/AcrA analyses, and protein analysis was performed with a 12% polyacrylamide gel. Polyclonal antipeptide antibodies directed against AcrA, OmpX, and OmpA were used as previously described (4, 14, 26). Evaluation of the OmpX expression levels was made as mentioned for AcrA protein, and *E. aerogenes* ATCC 13048(pJS04) was used as the positive strain.

Electrophoresis for detection of major *K. pneumoniae* porins was carried out with an SDS–11% polyacrylamide gel. Cell pellets prepared from both low- and high-osmolality media were used for immunodetection of major porins. The expression of OmpK35 porin has been reported to be downregulated by sorbitol; in contrast, OmpK36 is overexpressed in this high-osmolality medium (9). Our previous studies have shown a strong cross-immunoreactivity between *E. coli* and *K. pneumoniae* porins (28). Polyclonal antibodies directed against *E. coli* OmpC and OmpF porins have been used for the detection of main porins of *K. pneumoniae* strains (3, 28). The other conditions were the same as those described for OmpA, AcrA, and OmpX immunodetection. Evaluation of the OmpK35 and OmpK36 expressions was made by comparing the immunoblotting signals exhibited by the clinical strains and the control strains (*K. pneumoniae* Kp63 and *K. pneumoniae* ATCC 11296).

RESULTS

Antibiotic susceptibility testing. The 18 *K. pneumoniae* strains showed a significant degree of multiresistance according to the noticeable resistance level observed with various antibiotics (Tables 1 and 2). For ESBL-producing strains, aztreonam MICs ranged from 256 to ≥512 μg/ml, whereas for non-ESBL producers, the highest aztreonam MIC was 2 μg/ml (data not shown). The isolates could be divided into two major groups according to the results obtained with antibiotic susceptibility tests performed in the presence of the efflux pump inhibitor PAβN (Tables 1 and 2). A noticeable part (39%) of the *K. pneumoniae* collection exhibited a PAβN-sensitive resistance mechanism (Table 1). In group A isolates (Table 1) PAβN showed its significant effect on MICs of quinolones, chloramphenicol, and/or tetracycline. MICs were reduced by fivefold for at least one of these antibiotic classes tested with this efflux inhibitor (Table 1). Furthermore the strains in group A showed two kinds of susceptibility patterns against different types of quinolones. The MICs of all types of quinolones for K80 and K89 were similarly higher than those for other strains in this group, even when the measure was carried out in the presence of PAβN. For the remaining isolates (K2, K32, K33, K74, and K121) in this group, the MICs of quinolone were lower and the PAβN effect was particularly significant on ofloxacin MICs (Table 1). Concerning group B, the MICs of all antibiotics tested were generally higher for these isolates. In

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TABLE 2. Susceptibilities of *K. pneumoniae* isolates in group B to various antibiotics with and without an efflux pump inhibitor, PAβN

Strain	Source of strain	MIC (μg/ml)[a]										
		OFX	NOR	NAL	CHL	TET	FEP	CAZ	FOX	IPM	MEM	ESBL[e]
K41	Urine	128	≥512	≥512	≥512	16	4	128	64	0.5	≤0.06	+
K41 + PAβN[b]		16	256	≥512	64	4	4					
K17	Urine	64	512	≥512	≥512	≥128	≥512	≥512	64	0.25	≤0.06	+
K17 + PAβN		16	256	≥512	128	≥128	≥512					
K95	Urine	32	64	≥512	≥512	8	256	≥512	16	0.5	≤0.06	+
K95 + PAβN		4	64	≥512	128	4	256					
K100	Urine	32	512	≥512	≥512	≥128	256	≥512	32	0.5	2	+
K100 + PAβN		8	128	≥512	64	≥128	256					
K118	TA[c]	32	64	≥512	512	≥128	64	2	32	0.25	0.125	+
K118 + PAβN		4	16	256	32	≥128	8					
K116	Wound	32	64	≥512	≥512	≥128	32	2	32	0.5	≤0.06	+
K116 + PAβN		2	32	256	64	≥128	16					
K216	Urine	32	128	≥512	16	≥128	0.125	0.25	16	0.25	0.125	−
K216 + PAβN		8	128	512	2	≥128	ND[d]					
K192	Urine	32	256	≥512	512	≥128	0.5	0.25	8	0.25	≤0.06	−
K192 + PAβN		4	128	64	64	≥128	ND					
K104	Blood	4	32	≥512	256	≥128	0.125	0.125	4	1	≤0.06	−
K104 + PAβN		1	16	256	64	32	ND					
K128	Urine	4	32	≥512	4	4	≤0.06	0.25	4	0.5	≤0.06	−
K128 + PAβN		1	16	256	2	2	ND					
K132	Urine	4	16	≥512	≥512	≥128	≤0.06	0.25	16	1	≤0.06	−
K132 + PAβN		1	16	256	64	16	ND					

[a] Antibiotics were tested alone or with PAβN. MICs were determined in MH broth according to NCCLS guidelines (19). MICs were obtained from three independent measurements. Abbreviations: OFX, ofloxacin; NOR, norfloxacin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; FEP, cefepime; CAZ, ceftazidine; FOX cefoxitin; IPM, imipenem; MEM, meropenem.
[b] PAβN, 50 μM PAβN in each test tube.
[c] TA, tracheal aspiration.
[d] ND, not determined.
[e] ESBL, extended-spectrum β-lactamase.

addition, in this group the effect of PAβN on antibiotic susceptibility was strongly reduced compare to group A results (Tables 1 and 2).

Chloramphenicol accumulation test. In order to identify the presence of an efflux pump in PAβN-sensitive isolates, measurement of intracellular accumulation of chloramphenicol in the presence of two compounds, the efflux pump inhibitor PAβN and the membrane energy uncoupler CCCP, was performed (13, 20). Several strains belonging to groups A and B were tested for chloramphenicol accumulation capacity with and without PAβN and CCCP. Figure 1 illustrates the results obtained with clinical strains. In group A, K80 and K89 showed a PAβN-sensitive drug phenotype. A low level of intracellular accumulation of chloramphenicol was found in the absence of PAβN or CCCP in these isolates. In contrast, a three- to fourfold increase of intracellular drug concentration was obtained with inhibitors. In the PAβN nonresponding isolate, K128 (group B), the presence of the inhibitors during the incubation time did not lead to any significant change in chloramphenicol accumulation, and similar results were obtained with the ATCC 11296 strain (Fig. 1). These results indicated that an active efflux mechanism contributes to antibiotic resistance in isolates K80 and K89, which belong to group A, by decreasing the intracellular drug concentration. Activity of this efflux mechanism was significantly inhibited by the addition of PAβN or energy uncoupler.

Analyses of membrane protein profile. Hernández-Allés et al. have previously shown that sorbitol downregulates the expression of OmpK35 porin in *K. pneumoniae* (9). Consequently, we investigated the presence of porins in our isolates

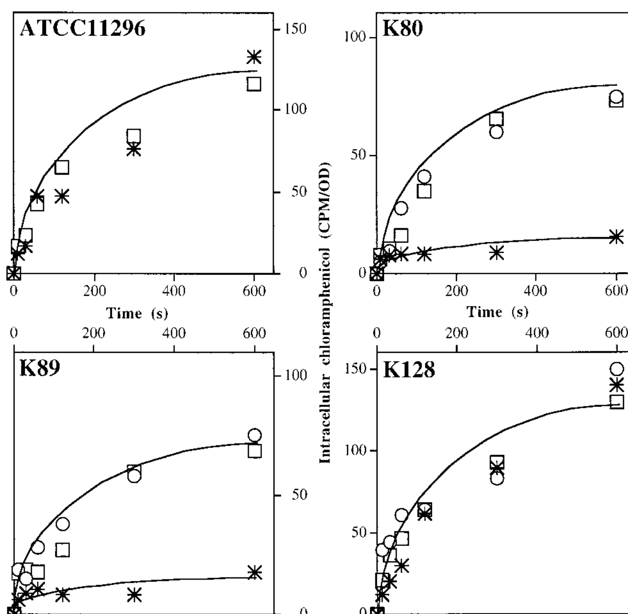

FIG. 1. Effect of efflux inhibitors on chloramphenicol intracellular accumulation in *K. pneumoniae* ATCC 11296 and isolates K80, K89, and K128. Exponential-phase bacteria in LB broth were removed, resuspended in sodium phosphate buffer, and incubated with radiolabeled chloramphenicol for various times. The experiments were carried out in the absence (✻) or in the presence (○) of CCCP or in the presence of PAβN (□). Values (expressed as counts per minute/optical density) were obtained from two independent experiments.

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TABLE 3. Expression of various proteins by *K. pneumoniae* strains[h]

Isolate	Expression of:						
	OmpA	AcrA	OmpK35		OmpK36		OmpX
			S^{-f}	S^{+g}	S^{-}	S^{+}	
Group A							
K80	+	+++	−	−	+	+	+
K89	+	+++	−	−	+	+	+
K2	+	+	±	−	+	+	+
K32	+	+++	±	−	+	+	+
K33	+	+	+	±	+	+	+
K74	+	+++	−	−	+	+	+
K121	+	++	−	−	+	+	+
Group B							
K41	+	++	−	−	+	+	+
K17	+	+	−	−	+	+	+
K95	+	±	−	−	+	+	+
K100	+	±	−	−	+	+	+
K118	+	+++	−	−	+	+	+
K116	+	+++	−	−	+	+	+
K216	+	+	−	−	+	+	+
K192	+	+	−	−	+	+	+
K104	+	+	+	−	+	+	+
K128	+	±	+	−	+	+	+
K132	+	+	+	−	+	+	+
Control strains							
ATCC 11296[a]	+	+	+	−	+	+	+
Kp 63[b]	+	ND[e]	−	−	−	−	ND
BW5104[c]	+	+	ND	ND	ND	ND	ND
ATCC 13048 (pJS04)[d]	+	ND	ND	ND	ND	ND	+++

[a] *K. pneumoniae* reference strain.
[b] *K. pneumoniae* 63 is the control strain used for immunodetection of porins (2).
[c] *E. coli* BW5104, the control strain used for AcrA immunodetection (26).
[d] *E. aerogenes*-type ATCC 13048 carrying pJS04 (*ompX* with *his* promoter); control strain used for OmpX immunodetection.
[e] ND, not determined.
[f] S^{-}, nutrient broth without sorbitol.
[g] S^{+}, nutrient broth with sorbitol.
[h] Expression of the various proteins was evaluated from immunodetection by Western blotting as previously described (4, 14). Symbols: +++, highly increased signal; ++, increased signal; +, normal signal; ±, weak signal; −, negative signal.

grown with and without sorbitol. A majority of the strains (12 of 18) did not express OmpK35, even in the low-osmolality medium (Table 3; Fig. 2). In contrast, the expression of OmpK36 porin was elevated in all isolates, and high-osmolality conditions did not modify its biosynthesis level.

Expression of OmpX has been previously reported to negatively regulate porin synthesis (29). Therefore, we determined its synthesis by immunodetection in our isolates. Interestingly, no significant variation of OmpX expression was detected in any of the strains (Table 3). Immunodetection of OmpA, which plays a key role in the conservation of the membrane architecture, was always positive (Table 3).

Concerning the efflux mechanism, we investigated the synthesis of AcrA, a component of the major pump in *Enterobacteriaceae*. The overproduction of this efflux protein has been previously reported in *E. aerogenes* clinical isolates that exhibit an antibiotic efflux mechanism (4, 26). In group A, five strains expressed a high level of AcrA, as illustrated in Fig. 2 and summarized in Table 3. Only two strains (K118 and K116)

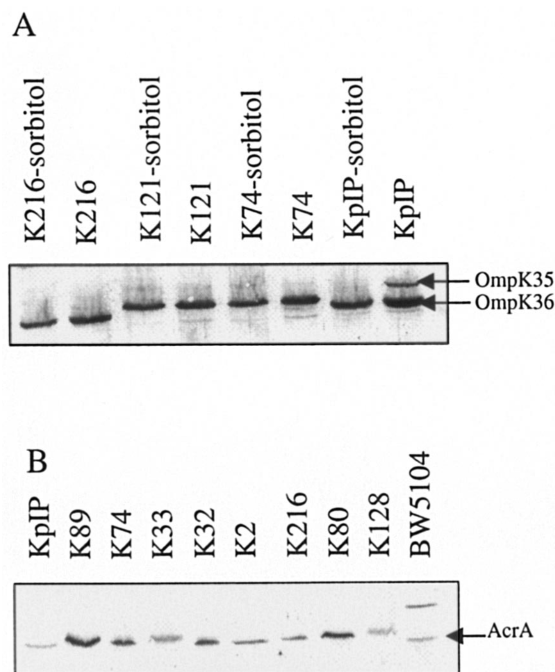

FIG. 2. Immunodetection of OmpK35, OmpK36, and AcrA in *K. pneumoniae* strains. Immunoblot assays of whole-cell extracts were carried out with antibodies directed against porins (A) or AcrA (B) as previously described (26, 28). Bacteria, including control strains, were grown with or without sorbitol for the analyses of porin expression. *K. pneumoniae* ATCC 11296 (KpIP) was used as the control strain in porin immunodetection. *E. coli* BW5104 (26), a strain expressing AcrA protein at a basal level, was used as a control strain in AcrA immunodetection. Only the relevant part of the immunoblots is shown.

belonging to group B exhibited an increased AcrA expression level (Table 2; Fig. 2).

DISCUSSION

Turkey is one of the countries with a high prevalence of the MDR phenotype in *K. pneumoniae* (8, 11, 23). In this study, we have investigated the role of an active drug efflux mechanism in the antibiotic resistance of eighteen MDR *K. pneumoniae* strains from that country. The activity of the drug efflux pump(s) was identified in 39% of the *K. pneumoniae* strains. A significant decrease in MICs of quinolones, chloramphenicol, and/or tetracycline was obtained with an efflux pump inhibitor, PAβN, in these strains (Table 1). Concerning group A, we observed two different quinolone susceptibility patterns and also two kinds of response to the PAβN effect. The effect of the efflux inhibitor on high MICs of quinolone for two strains (K80 and K 89) in this group was significant but it was not sufficient to reduce these MICs into the sensitivity ranges. This is the case for norfloxacin and nalidixic acid (Table 1). Mutations in the target proteins, DNA gyrase and topoisomerases IV, are probably involved in the quinolone resistance of those isolates in addition to the expression of efflux pump machinery. PAβN′s effect on the MICs of quinolone for the other strains (K2, K32, K33, K74, and K121) in group A was more significant and sufficient to restore a susceptible profile particularly for nalidixic acid. This result suggested that the major quino-

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lone resistance mechanism found in those strains is a PAβN-sensitive mechanism, namely, a drug efflux mechanism. The results of MICs of drugs plus PAβN were confirmed by chloramphenicol accumulation tests carried out with PAβN or CCCP for certain group A strains (K80 and K89). A large increase in the intracellular concentration of chloramphenicol was generated by the uncoupler CCCP and by the pump inhibitor PAβN in those two strains. In the strain K128 from group B, for which the MICs were nonsensitive to PAβN, no variation in the level of chloramphenicol accumulation was observed after the treatment with these efflux inhibitors. On the basis of these results, we may conclude that the PAβN-sensitive efflux pump system detected in the strains from group A depends on the proton motive force, which is collapsed by the energy uncoupler (20). On the other hand, the high expression level of AcrA protein observed in most of the isolates belonging to group A (five of seven strains) is strong evidence for overexpression of the AcrAB-mediated efflux mechanism in those isolates. This overproduction strongly contributes to their multiple-antibiotic resistance. In *E. aerogenes*, we have shown previously that the AcrAB/TolC complex participates in chloramphenicol, quinolone, and tetracycline efflux (4, 26) and this pump is inhibited by CCCP and PAβN (4, 14, 15). The results of this study correlated well with recent reports indicating the role of AcrA in ciprofloxacin resistance of *K. pneumoniae* isolates (18, 27). Taking into account these data and the results presented here, AcrAB/TolC may be the major efflux machinery functioning in MDR *K. pneumoniae* clinical isolates. It is also important to note that all strains overexpressing the AcrA component also presented alterations in their porin profile and none of them expressed OmpK35 porin in both high- and low-osmolality culture media. On the other hand, OmpK35 deficiency was also detected in most of the other strains, including ESBL producers, in our *K. pneumoniae* collection. The other major porin, OmpK36, was expressed by all isolates. In all clinical isolates, no variation was detected in OmpX and OmpA synthesis. These observations suggested the absence of a pleitropic alteration impairing the outer membrane protein expression or assembly in these strains (14). Taking into account the simultaneous overproduction of AcrA and alteration of the porin profile, we propose that the complex *marA* genetic cascade induces the decrease of porin expression via special regulation, such as that of *micF*, and the activation of efflux pump synthesis in these strains (1, 5, 6, 21, 27).

Finally, the efflux pump mechanism, AcrAB/TolC, significantly contributes to antibiotic resistance in our *K. pneumoniae* strains. Interestingly, overexpression of efflux pump machinery has been recently shown among nosocomial *K. pneumoniae* strains during a hospital outbreak (7). Further epidemiologic surveys are necessary to better understand the prevalence of efflux pump activation in the emergence of an MDR phenotype in *K. pneumoniae*, and the PAβN protocol may be a good indicator in the screening of efflux pump activation.

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