AcrAB Efflux System: Expression and Contribution to Fluoroquinolone Resistance in *Klebsiella* spp.

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Seven *Klebsiella pneumoniae* and four *Klebsiella oxytoca* clinical isolates with different levels of resistance to ciprofloxacin were studied. Mutations in the topoisomerase genes were found in almost all strains, but the contribution of a multidrug efflux system homologous to AcrAB in *Escherichia coli* was also observed. Overexpression of this efflux system was demonstrated by immunoblotting with antibodies against *E. coli* AcrA.

In recent years, an increasing number of fluoroquinolone-resistant strains, especially of *Escherichia coli*, have been isolated from outpatients (3). Mutations in a small region at the beginning of the *gyrA* gene, called the quinolone resistance-determining region (QRDR), are often associated with the resistance phenotype. Recently, an additional mechanism of quinolone resistance, involving an active efflux system, has been found in *E. coli* strains with high levels of resistance and associated with both multiple mutations in the topoisomerase genes and overexpression of the AcrAB multidrug efflux system (2, 8, 11). This dual contribution to fluoroquinolone resistance has been demonstrated also for other organisms, such as *Pseudomonas aeruginosa* (4, 7), *Staphylococcus aureus* (6), and *Streptococcus pneumoniae* (5).

Resistance phenotypes are associated with mutations in the QRDRs of *Klebsiella pneumoniae* and *Klebsiella oxytoca*, too (1, 13), and the presence of the AcrAB operon in a clinical strain of *K. pneumoniae* was recently demonstrated (A. Domenech-Sanchez, S. Alberti, L. Martinez-Martinez, A. Pascual, I. Garcia, and V. J. Benedi, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-2018, p. 104, 2001), although its role in fluoroquinolone resistance was not discussed in this preliminary report.

Seven ciprofloxacin-resistant clinical strains of *K. pneumoniae* and four of *K. oxytoca*, isolated at the Verona University Hospital from August 1996 to October 1998, were studied. One ciprofloxacin-susceptible strain per species and *E. coli* AG100, which expresses normal levels of the AcrAB system (12), were also included. The MICs of novobiocin, ciprofloxacin, and nalidixic acid were tested by an agar dilution method, according to NCCLS guidelines (10), and the results are reported in Table 1. Novobiocin was included because it is a good substrate for the AcrAB efflux system of *E. coli*. The ciprofloxacin MICs for resistant strains ranged from 2 to 64 µg/ml, but only one strain (KLPN 86) showed a high level of resistance (MIC = 64 µg/ml). Sequencing of the QRDRs of *gyrA* and *parC* was carried out with all *K. pneumoniae* and *K. oxytoca*

strains, by using conditions previously reported (1, 13). Mutations at codon 83 of *gyrA* in six of the seven resistant *K. pneumoniae* strains were found (Table 1). In five cases, the mutation at codon 83 was a C→A transversion in the codon TCC, resulting in the substitution of tyrosine for serine. In one strain, the mutation at codon 83 was a C→T transversion, resulting in a phenylalanine substitution for serine. Strain KLPN 105 showed the wild-type sequence. Mutations were also found in the QRDR region of *parC* in two resistant strains. In one case (strain KLPN 86) the mutation was at codon 80 and was a G→T transversion, resulting in the substitution of isoleucine for serine; in the other strain (KLPN 87) the mutation was at codon 79 and was an A→G transversion, resulting in the substitution of glycine for aspartic acid. In all resistant *K. oxytoca* strains, the mutation resulting in substitution of isoleucine for threonine at codon 83 was found in the *gyrA* gene. No mutations were found in the QRDRs of the *gyrA* and *parC* genes of the two ciprofloxacin-susceptible klebsiellae.

To assess the presence of the AcrAB efflux system, *acrA* was amplified by using the primers KLACRA/FW (5′ ACTCGAG GTTTACAAATG) and KLACRA/R (5′ AGGCATGTCTTA ACGGCT), derived from the published sequence (accession number AJ318073). The PCR conditions were 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min for 30 cycles. The sequence of the 1.2-kb amplicons of the *acrA* gene from all isolates, both resistant and susceptible, was determined and compared with *acrA* of the deposited *K. pneumoniae* sequence (Domenech-Sanchez et al., 41st ICAAC). The *acrA* gene of our strains showed nucleotide identities ranging from 91.5 to 97.9% for *K. pneumoniae* and from 82 to 85.1% for *K. oxytoca*. The identities at the protein level ranged from 94.2 to 95.9% for *K. pneumoniae* and from 90.2 to 90.4% for *K. oxytoca*. In our strains, one amino acid (Q175) was always missing and most differences were found in a region of nine amino acids, from position 177 to position 185. In *K. pneumoniae* strains, other common differences from the deposited sequence were F92S, G103A, N153S, T162A, and V210L.

Using the available anti-AcrA antibodies (14), immunoblotting was employed to assess the correlation between fluoroquinolone resistance and expression of the AcrAB efflux system, according to the procedure already described (8). The strains were grown in Luria-Bertani broth at 37°C until the

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TABLE 1. Antimicrobial susceptibilities, QRDR mutations, AcrA expression levels, and ciprofloxacin accumulation in clinical strains of *K. pneumoniae* and *K. oxytoca*

Strain	MIC (μg/ml)			QRDR mutation		AcrA expression level[b] (mean ± SD)	Level of ciprofloxacin accumulation[c] without CCCP	With CCCP
	Ciprofloxacin	Nalidixic acid	Novobiocin	*gyrA*	*parC*			
E. coli AG100[a]	0.03	4	256	None	None	1.0[b]	1.0[c]	1.0[c]
K. pneumoniae								
KLPN 42	4	>512	256	S83Y	None	1.0 ± 0.047	0.83	0.95
KLPN 86	64	>512	>512	S83F	S80I	1.24 ± 0.051	0.42	1.08
KLPN 87	4	>512	16	S83Y	D79G	0.9 ± 0.07	0.85	1.1
KLPN 90	4	>512	128	S83Y	None	1.23 ± 0.03	0.55	1.1
KLPN 94	0.03	4	64	None	None	0.9 ± 0.15	1	1.1
KLPN 96	4	>512	128	S83Y	None	1.31 ± 0.11	0.55	1.0
KLPN 103	2	>512	64	S83Y	None	1.0 ± 0.045	0.42	0.87
KLPN 105	4	>512	64	None	None	1.7 ± 0.15	0.35	0.83
K. oxytoca								
KLOXY 106	4	>512	256	T83I	None	2.2 ± 0.25	0.50	0.83
KLOXY 108	2	>512	256	T83I	None	2.2 ± 0.22	0.43	0.97
KLOXY 109	2	>512	256	T83I	None	1.65 ± 0.16	0.36	0.85
KLOXY 114	0.03	4	16	None	None	0.53 ± 0.1	1.20	1.36
KLOXY 116	4	>512	128	T83I	None	1.14 ± 0.03	0.73	0.96

[a] Wild-type strain that expresses a normal level of the AcrAB efflux system.
[b] The levels of expression of the AcrA protein were determined as described in the text and were expressed relative to that for *E. coli* AG100. Tests were repeated three times.
[c] The accumulation of ciprofloxacin (in nanograms per milligram of cells) was determined as described in the reference 9 and was expressed relative to that for *E. coli* AG100. CCCP was used at 100 μM.

culture attained a density of about 0.2 mg (dry weight) ml^{-1}. The cells were harvested by centrifugation and resuspended in the same volume of 1% sodium dodecyl sulfate, followed by heating in a boiling water bath for 10 min. Whole-cell extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel. Proteins were electroblotted onto nitrocellulose membranes and probed with polyclonal antibodies raised against *E. coli* AcrA, and the immunoblots were developed with the ECL Western blotting detection reagent (Amersham Pharmacia). The intensities of the bands obtained were compared by using ImageMaster VDS analysis software (Pharmacia Biotech). In each experiment, *E. coli* AG100, a reference strain with a normal level of expression, was included as a standard, and the AcrA levels in various strains were calculated as relative values in comparison with that for AG100. The experiments were repeated three times, and average values are reported in Table 1. Four of seven *K. pneumoniae* strains (including strain KLPN 105) showed overexpression of AcrA protein, at levels up to 130% of that of the control strain, *E. coli* AG100. The other three resistant
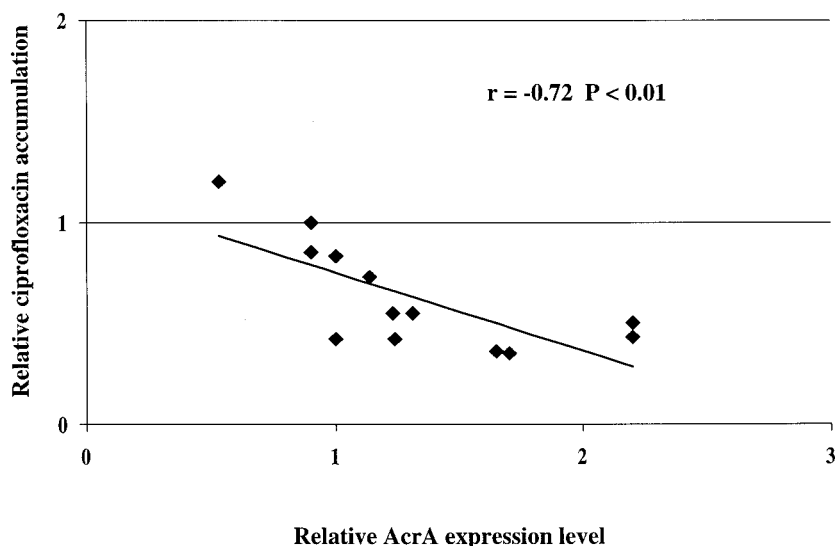

FIG. 1. Correlation of AcrA expression and ciprofloxacin accumulation. The relative level of AcrA expression and the relative ciprofloxacin accumulation are reported in Table 1.

K. pneumoniae strains and the susceptible strain of the same species showed AcrA levels ranging from 90 to 100% of that of the control. All *K. oxytoca* ciprofloxacin-resistant strains overproduced the AcrA protein, with levels ranging from 114 to 220% of that of the control. The susceptible strain of the same species had a reduced expression of AcrA (roughly 50% of that of AG100).

Ciprofloxacin accumulation in bacterial cells, in the presence or absence of the energy inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), was measured by the method of Mortimer and Piddock (9). A reduced ciprofloxacin accumulation, ranging from 15 to 65%, was found in all ciprofloxacin-resistant *K. pneumoniae* strains. This accumulation was reversed in all cases by addition of CCCP. In all cases, the ciprofloxacin-resistant *K. oxytoca* strains also showed a CCCP-reversible reduction (50% or less) of ciprofloxacin accumulation.

All strains of *K. pneumoniae* and *K. oxytoca* examined in our study contained sequences which showed a high degree of homology with the AcrA sequence of *K. pneumoniae* described previously, and a significant correlation ($r = -0.72$; $P < 0.01$) between AcrA expression and ciprofloxacin accumulation was observed (Fig. 1). The three resistant *K. pneumoniae* strains with normal expression of AcrA showed a lower uptake of ciprofloxacin than the reference strain, suggesting the possible involvement of efflux systems other than AcrAB. With only one exception (KLPN 105), resistance was also associated with mutations in the *gyrA* QRDR in all strains. Only two strains (KLPN 86 and KLPN 87) had mutations in the *parC* QRDR. All mutations were of previously described types (1, 13) except for D79G in *parC* of strain KLPN 87.

The contribution of efflux system overexpression to fluoroquinolone resistance was clearly demonstrated both by the behavior of strain KLPN 105, which did not carry any mutations in *gyrA* and *parC* but showed the highest level of AcrA expression, and by the two strains with mutations in both *gyrA* and *parC*, of which only the one overexpressing AcrA (KLPN 86) showed a high level of resistance.

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