ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2003, p. 2831–2837 Vol. 47, No. 9
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.9.2831–2837.2003

Role of AcrR and RamA in Fluoroquinolone Resistance in Clinical *Klebsiella pneumoniae* Isolates from Singapore

T. Schneiders,[1] S. G. B. Amyes,[2] and S. B. Levy[1,3]*

Department of Molecular Biology and Microbiology[1] and Department of Medicine,[3] Center for Adaptation Genetics and Drug Resistance, Tufts University School of Medicine, Boston, Massachusetts 02111, and University of Edinburgh Medical School, Edinburgh EH8 9AG, United Kingdom[2]

Received 5 February 2003/Returned for modification 7 April 2003/Accepted 5 June 2003

The MICs of ciprofloxacin for 33 clinical isolates of *K. pneumoniae* resistant to extended-spectrum cephalosporins from three hospitals in Singapore ranged from 0.25 to >128 μg/ml. Nineteen of the isolates were fluoroquinolone resistant according to the NCCLS guidelines. Strains for which the ciprofloxacin MIC was ≥0.5 μg/ml harbored a mutation in DNA gyrase A (Ser83→Tyr, Leu, or Ile), and some had a secondary Asp87→Asn mutation. Isolates for which the MIC was 16 μg/ml possessed an additional alteration in ParC (Ser80→Ile, Trp, or Arg). Tolerance of the organic solvent cyclohexane was observed in 10 of the 19 fluoroquinolone-resistant strains; 3 of these were also pentane tolerant. Five of the 10 organic solvent-tolerant isolates overexpressed AcrA and also showed deletions within the *acrR* gene. Complementation of the mutated *acrR* gene with the wild-type gene decreased AcrA levels and produced a two- to fourfold reduction in the fluoroquinolone MICs. None of the organic solvent-tolerant clinical isolates overexpressed another efflux-related gene, *acrE*. While *marA* and *soxS* were not overexpressed, another *marA* homologue, *ramA*, was overexpressed in 3 of 10 organic solvent-tolerant isolates. These findings indicate that multiple target and nontarget gene changes contribute to fluoroquinolone resistance in *K. pneumoniae*. Besides AcrR mutations, *ramA* overexpression (but not *marA* or *soxS* overexpression) was related to increased AcrAB efflux pump expression in this collection of isolates.

Klebsiella pneumoniae is a common cause of nosocomial infections that include urinary tract, respiratory, and wound infections. *Klebsiella* spp. have been found to harbor a multitude of plasmids, which confer resistance to most β-lactams, particularly the extended-spectrum cephalosporins and, more recently, the carbapenams (33). These emerging resistance mechanisms have dictated a shift in the strategies used to treat infections caused by *Klebsiella* spp. with the fluoroquinolones (3, 29, 32). Recent studies indicate that an increasing percentage of *Klebsiella* species are resistant to these antimicrobials (3).

Fluoroquinolone resistance arises through specific mutations within the target proteins DNA gyrase and topoisomerase IV, more specifically, within a region termed the quinolone-resistance determining region (2, 5, 6). In members of the family *Enterobacteriaceae*, the most common changes occur at position 83 or 87 within DNA gyrase A and position 80 or 84 within the ParC subunit of topoisomerase IV (2, 5, 6). Mutations at all the positions mentioned above have been described in fluoroquinolone-resistant *K. pneumoniae* isolates (2, 5, 6, 21, 23), and a fifth mutation at position 78 in *parC* has recently been identified (21).

Besides topoisomerase mutations, energy-dependent efflux and porin loss have also been shown to confer a fluoroquinolone resistance phenotype in *K. pneumoniae* (4, 11, 21, 22). These two changes often occur together in the majority of multidrug-resistant *Klebsiella* isolates (11, 19, 21). The latter finding may reflect the activities of chromosomal regulatory loci like *mar* and *sox*, which cause decreased porin expression and increased efflux pump expression (13, 17, 26). The effects of these loci generally require a mutation within the repressor genes of the operons or the selective binding of certain inducers like salicylate (1) and paraquat (36). Mutations within MarR, the negative regulator of the *mar* operon, cause overexpression of *marA* in the *Enterobacteriaceae*, e.g., *Escherichia coli* (18, 24, 26, 28, 35) and *Enterobacter cloacae* (17), resulting in an antibiotic resistance phenotype. Similarly, mutations within *soxR* can lead to *soxS* overexpression, which also results in both organic solvent tolerance and antibiotic resistance (15, 35).

Unlike *E. coli*, *K. pneumoniae* encodes another transcriptional activator, RamA, which can confer a multidrug resistance phenotype when it is overexpressed in *E. coli* (7). RamA is thought to form an operon with a gene specifying the outer membrane protein RomA. RamA acts as a transcriptional activator, while RomA is a putative channel-forming protein (7, 14) somehow involved in the resistance phenotype. The role of *ramA* in the antibiotic resistance phenotype of clinical strains has not been described.

Studies performed with *E. coli* (13, 18, 26), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (15, 31), and *E. cloacae* (17) implicate both *mar* and *sox* in fluoroquinolone resistance phenotypes through the overexpression of the multidrug efflux pump AcrAB. Mutations within the repressor (AcrR) have also been shown to lead to *acrA* (34) and *acrB* (35) overexpression. Complementation of the mutated *acrR* with the wild-type gene was shown to decrease the level of antibiotic resistance in *E. coli*, implicating the role of a functional repressor in controlling the highly drug resistant pheno-

* Corresponding author. Mailing address: Center for Adaptation Genetics and Drug Resistance, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6764. Fax: (617) 636-0458. E-mail: stuart.levy@tufts.edu.

TABLE 1. Laboratory strains and plasmids used in the study

Strain or plasmid	Description	Reference
Strains		
AG100	Wild-type *E. coli* K-12 strain	8
AG112	MarR mutant of AG100; 5-bp deletion (from positions 1481 to 1485)	25
AG100A	AG100 Δ*acrAB*	27
AG100B	AG100 *acrR*::Kan mutant	27
DJ901	*E. coli* GC4468 *soxRSΔ901*::Tn*10*Kan	9, 10
JTG1078	GC4468 *soxR105 zjc-2204*::Tn*10*kan	10
KP3	*K. pneumoniae,* fluoroquinolone-susceptible isolate from Bacteriology Department, NEMC[a]	This study
Plasmids		
pSHA2	Tellurite resistance determinant with OmpK36 *Klebsiella* porin in pACYC184	20
pTS003	Tell[r] Chl[r] in pACYC184[b]	This study
pTS003*acrR*	pTS003 with *K. pneumoniae acrR*	This study

[a] NEMC, New England Medical Center, Boston, Mass.
[b] Tell[r], potassium tellurite resistance determinant.

type (34). Other efflux pumps, such as AcrEF, have also been shown to mediate resistance in laboratory mutants of *E. coli*, particularly in the absence of a functional AcrAB efflux pump (12). The contribution of AcrEF to a resistance phenotype in clinical isolates has not been described. In fluoroquinolone-resistant *K. pneumoniae* isolates, a correlation between reduced levels of fluoroquinolone uptake and AcrA overexpression has been observed, although the genetic basis of this overexpression was not described (23).

This paper investigates the roles of the *mar*, *sox*, and *ram* loci and the efflux pumps AcrAB and AcrEF in the production of the ciprofloxacin resistance phenotype observed in clinical isolates of *K. pneumoniae* resistant to extended-spectrum cephalosporins.

MATERIALS AND METHODS

Bacterial strains. Thirty-three clinical *K. pneumoniae* strains (provided by R. Lin, Kandang Kerbau Hospital, Singapore), selected on the basis of resistance to extended-spectrum cephalosporins, were recovered from the following centers: Kandang Kerbau Hospital (23 isolates), Singapore General Hospital (9 isolates), and Alexandra Hospital (1 isolate). The identities of these clinical isolates were reconfirmed by testing with an Analytab Products system prior to further manipulation. The laboratory strains used in this study are described in Table 1.

MICs. Testing of susceptibilities to ciprofloxacin, moxifloxacin (Bayer AG, Wuppertal, Germany), gatifloxacin (Grunethal, GmbH, Anchen, Germany), and

TABLE 2. Primers and annealing temperatures used in the study

Gene	Primer	T_m (°C)[a]	GenBank Accession no.
KpgyrA1	5′-TGCGAGAGAAATTACACC-3′	56	X16817
KpgyrA2	5′-AATATGTTCCATCAGCCC-3′		
KpmarR1	5′-CCAGCGACCTGTTTAATGA-3′	56	M96235
KpmarR2	5′-GCGTCATTATTACGTCTGG-3′		
KpmarA1	5′-TGCTCAAGAAGGTCCTGCC-3′	58	M96235
KpmarA2	5′-TGCGGCAGCGAATAGTTTC-3′		
KpsoxS1	5′-CCCATCAGGATATTATTCA-3′	52	U00734
Kpsox2	5′-AGATGTGATGGCGATAGT-3′		
KpacrE1	5′-ATGACGACTCACGCCA-3′	50	M96848
KpacrE2	5′-CGTTTCACCGTCAAATG-3′		
KpramA1	5′-GGGTCGCCGATAAGACGC-3′	60	U19581
KpramA2	5′-GCTGGGCGCCATTGAGTAT-3′		
Kpramop1	5′-TATCAACGGCTGGCGGCT-3′	58	U19581
Kpramop2	5′-GCAGCGGTTGATGCAGGT-3′		
KpacrR*Nru1*[b]	5′-TGAGTCGCGAATTAAGCTGACAAGCTCTC-3′	58	AJ318073
KpacrR*Bcl1*[c]	5′-TGAGTGATCAGGTCATGCTATGGTACATA-3′		
KpacrA1	5′-ATGAACAAAAACAGAGG-3′	52	AJ318073
KpacrA2	5′-TTTCAACGGCAGTTTTCG-3′		

[a] T_m, melting temperature.
[b] Underlining indicates the *Nru*1 site.
[c] Underlining indicates the *Bcl*1 site.

TABLE 3. Topoisomerase mutations and OST in selected clinical isolates of *K. pneumoniae*

Isolate[a]	Ciprofloxacin MIC (μg/ml)	Amino acid at DNA gyrase A position:		Amino acid at topoisomerase IV position 80	OST[b]
		83	87		
S8	0.5	Ser→Tyr	—[c]	—	−
S38	2	Ser→Tyr	—	—	−
S28	4	Ser→Tyr	—	—	C, P
S29	4	Ser→Leu	Asp→Asn	—	C, P
S30	8	Ser→Tyr	—	—	C
S37	8	Ser→Tyr	—	—	C
S7	16	Ser→Tyr	—	Ser→Ile	C, P
S9	16	Ser→Tyr	Asp→Asn	Ser→Trp	−
S12	16	Ser→Ile	Asp→Asn	Ser→Ile	−
S10	32	Ser→Tyr	Asp→Asn	Ser→Arg	C
S27	32	Ser→Tyr	Asp→Asn	Ser→Ile	−
S33	32	Ser→Tyr	Asp→Asn	Ser→Ile	−
S34	32	Ser→Ile	—	Ser→Ile	−
S5	64	Ser→Tyr	Asp→Asn	Ser→Ile	C
S13	64	Ser→Tyr	Asp→Asn	Ser→Trp	C
S31	64	Ser→Tyr	Asp→Asn	Ser→Trp	−
S42	64	Ser→Tyr	—	—	−
S6	128	Ser→Tyr	Asp→Asn	Ser→Arg	C
S32	128	Ser→Tyr	—	Ser→Trp	−
S36	128	Ser→Tyr	Asp→Asn	Ser→Trp	C

[a] Isolates are listed by increasing ciprofloxacin MIC.
[b] C, resistant to cyclohexane; P, resistant to pentane; −, no growth in the presence of cyclohexane or pentane.
[c] —, no change at specified amino acid.

gentamicin (Sigma, Poole, United Kingdom) was performed by the doubling agar dilution method as described in the guidelines of the British Society for Antimicrobial Chemotherapy (30). The fluoroquinolone susceptibilities of the clinical strains with and without plasmids pTS003 or pTS003*acrR* were determined on Luria-Bertani agar by E-test (AB Biodisk, Solna, Sweden) or doubling agar dilution at 37°C overnight for 18 h.

PCR. Primers specific for the quinolone-resistance determining region were designed and used in the amplification of both the *gyrA* and the *parC* regions (5). Primers specific for the *marR*, *marA*, *soxS*, and *acrE* genes were obtained from the genome sequence of *K. pneumoniae* (http://genome.wustl.edu/projects/bacterial/) by comparison with the homologous *marRA* (GenBank accession no. M96235), *soxS* (GenBank accession no. U00734), and *acrE* (GenBank accession no. M96848) genes of *E. coli* with the BLAST program. Primers specific for the *acrR* and *acrA* sequences were obtained from the National Center for Biotechnology Information (GenBank accession no. AJ318073) and the *K. pneumoniae* genome sequence (http://genome.wustl.edu/projects/bacterial/). The sequences of all primers used in the study are listed in Table 2. Genomic DNA was extracted by using the Tissue Amp kit from Qiagen, Inc., and was used as the template for all PCRs. All PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Inc.) according to the guidelines of the manufacturer. Bidirectional sequencing of all PCR products was performed to confirm the mutations and the presence of the cloned genes. Sequencing of the *gyrA* and *parC* products was performed at the Department of Hematology, Royal Infirmary of Edinburgh; *acrR*, *marR*, and *ram* operator and promoter amplimers were sequenced at the Tufts University Core Facility.

OST. Organic solvent tolerance (OST; tolerance of hexane and pentane [Sigma-Aldrich Chemical Co., Milwaukee, Wis.] and cyclohexane [Fisher Scientific]) was determined as described previously (34). The plates were incubated at 30°C for 24 h before they were scored for growth. Strains AG100 and AG112 were used as negative and positive controls, respectively (34).

Northern blotting analysis. The RNAeasy Bacterial kit (Qiagen, Inc.) was used to extract RNA, with 1 to 5 μg of RNA separated by electrophoresis on a 1% formaldehyde agarose gel. Hybridization was carried out with DNA probes labeled with [α-^{32}P]dCTP (New England Nuclear, Worcester, Mass.) according to the instructions of the manufacturer (Invitrogen Life Technologies, Carlsbad, Calif.). For Northern blotting analysis of *marA*, AG100 and AG112 (a *marR* mutant) served as controls; for Northern blotting analysis of *soxS*, DJ901 (a strain from which *soxS* was deleted) and JTG1078 (a *soxS*-overexpressing strain) were used as controls. For Northern blot analysis of *acrA*, AG100A (from which *acrAB* was deleted) and AG100B (an *acrR* mutant) were used as controls.

Construction of *acrR*-complementing plasmid. The 0.7-kb *acrR* fragment was amplified from the genomic DNA of a susceptible *Klebsiella* isolate, isolate KP3, provided by the Bacteriology Department of the New England Medical Center, Boston, Mass. (Table 1). Sequence analysis confirmed that the AcrR region possessed 96% amino acid identity to the sequence in the GenBank database (GenBank accession no. AJ318073) (see Table 4). This fragment was then restricted with *Nru*I and *Bcl*I and ligated to pACYC184, which had previously been digested with the same enzymes. The vector pSHA2 (20), which harbors the potassium tellurite resistance gene, was digested with *Not*I to release the 3-kb resistance cassette, which was then ligated to *Eag*I-digested pACYC184, transformed into DH5α cells, and selected on potassium tellurite (25 μg/ml) and chloramphenicol (30 μg/ml), thereby creating plasmid pTS003*acrR*. Potassium tellurite resistance was used because of the multiresistance phenotype of the clinical bacteria, which excluded the possibility of selection with conventional antibiotics. The cloned wild-type *acrR* gene was transferred into the clinical isolates in which an *acrR* mutation had been detected by electroporation of pTS003*acrR*. The vector-only control was constructed by ligating the *Not*I-restricted 3-kb potassium tellurite resistance fragment from pSHA2 to *Eag*I-digested pACYC184 to form pTS003. The effect of the cloned wild-type *acrR* was examined by determining the antibiotic susceptibilities in comparison to those of the vector-only controls and expression of AcrA (by Western blotting).

Western blotting analysis. *K. pneumoniae* isolates with the *acrR*-containing plasmid and the corresponding wild-type strains were freshly grown in Luria-Bertani broth to an A_{600} of 0.8. Twenty micrograms of total protein was loaded for the detection of AcrA, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 15% gel as described previously (16). All further manipulations were performed as described previously (34). Briefly, the membrane was blocked overnight at room temperature with 5% dried milk and hybridized with anti-AcrA polyclonal antibody (1:8,000; gift from H. Zgurskaya, University of Oklahoma, Norman) at room temperature for 1 h with shaking. After three washes in wash buffer, the membrane was incubated at room temperature for 1 h with horseradish peroxidase conjugated to anti-rabbit immunoglobulin G (1:2,000; Life Technologies) diluted in wash buffer. The blots were developed with Renaissance Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Inc., Boston, Mass.). Control strains AG100A (from which *acrAB* was deleted) and AG100B (an *acrR* mutant with AcrA overexpression) were used to assess the relative amounts of AcrA. Densitometric analysis of the relative AcrA values for the clinical strains was performed both before and after complementation with the wild-type *acrR*. The levels of AcrA expression were quantified by using the National Institutes of Health Image Program (http://rsb.info.nih.gov/nih-image/manual/index.html).

RESULTS

Antibiotic and organic solvent susceptibilities of *K. pneumoniae* isolates. Of the 33 clinical isolates tested, 19 were

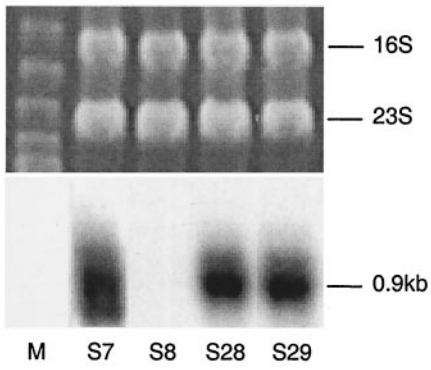

FIG. 1. Northern blotting analysis of *ramA* expression in *Klebsiella* isolates. *K. pneumoniae* strains (S7, S28, and S29) overproduced *ramA* mRNA in comparison to the level of production by S8 (an organic solvent-susceptible and fluoroquinolone-susceptible isolate). Northern blotting analysis with a *ramA*-specific probe revealed mRNA of 0.9 kb, signifying a polycistronic message. Lane M, molecular size marker.

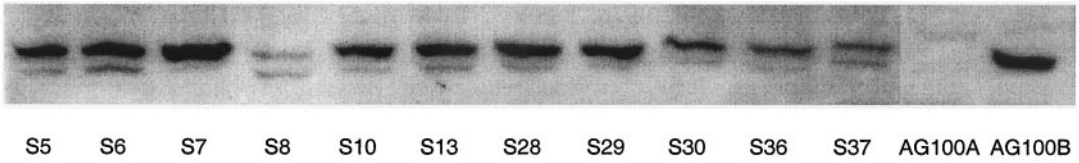

FIG. 2. Western blotting analysis of AcrA expression by *Klebsiella* isolates (all isolates except S8 were organic solvent tolerant) as well as AG100A (from which *acrAB* was deleted and which did not express AcrA) and AG100B (an AcrR mutant with AcrA overexpression). Representative results of analyses performed twice are shown.

resistant to fluoroquinolones, with MICs being greater than 4 μg/ml (NCCLS guidelines) (Table 3). The efficacies of the newer fluoroquinolones (e.g., gatifloxacin and moxifloxacin) were comparable to those of ciprofloxacin. Twenty-four isolates were resistant to gentamicin according to the NCCLS guidelines (MICs, ≥16 μg/ml). While all 33 isolates grew well in the presence of hexane, 10 of the 19 fluoroquinolone-resistant strains were resistant to cyclohexane and 3 were resistant to pentane (Table 3). The OST phenotype observed for the 10 clinical isolates was not associated with resistance to a particular fluoroquinolone (Table 3).

Mutations in DNA gyrase A and ParC. The topoisomerase mutations in 20 selected clinical strains for which ciprofloxacin MICs were ≥0.5 μg/ml (Table 3) were generally found at Ser83 or Ser87 in GyrA and at Ser80 in ParC. The most common mutation (in strains for which ciprofloxacin MICs were ≥0.5 μg/ml) was Ser83→Tyr in GyrA; this change was present in 17 of 20 isolates tested. The acquisition of the mutation Asp87→Asn in GyrA, observed in 11 of 20 strains, was generally associated with an increase in the fluoroquinolone MICs and was also the only amino acid substitution observed at position 87. Mutations in ParC, present in 13 of 20 isolates, occurred only at position 80 and were most commonly Ser80→IIe. In two of the isolates (isolates S34 and S32), the ParC change of Ser80→Trp or IIe occurred without a change in *gyrA* at position 87. None of the different amino acid substitutions was associated with a particular fluoroquinolone MIC. Additionally, there was no correlation between the topoisomerase mutations, OST, and the fluoroquinolone MIC.

Expression of *marA*, *soxS*, and *ramA*. The overexpression of *marA*, *soxS*, and *ramA* in the 10 isolates with OST and 1 isolate (isolate S8) susceptible to fluoroquinolones and organic solvents was determined by Northern blotting analysis. Of the 11 isolates investigated, none produced a transcript indicating *marA* or *soxS* overexpression (data not shown). Sequence analysis revealed no mutations within the *marR* region, confirming the results of the Northern blotting analysis. *ramA* overexpression was found in three organic solvent-tolerant isolates (isolates S7, S28, and S29) (Fig. 1). Sequence analysis showed that overexpression was not related to mutations within the *ram* operator or promoter region.

Expression of *acrA* and *acrE*. There was a strong correlation between the results of Northern blotting analysis of the organic solvent-tolerant isolates and those of Western blotting analysis with anti-AcrA antibody (Fig. 2) for all except two strains (strains S6 and S28). These strains did not exhibit detectably increased levels of *acrA* expression by Northern blotting but produced elevated levels of AcrA (Fig. 2). As expected, organic solvent- and fluoroquinolone-susceptible isolate S8 expressed low, albeit detectable, levels of AcrA (Fig. 2). None of the clinical isolates overproduced another efflux-related gene, *acrE*, as assayed by Northern blotting with the same RNA samples.

Genetic analysis of AcrR. Of the 10 strains sequenced and compared to the isolate in the GenBank database (GenBank accession no. AJ318073), one group of 5 strains (strains S6, S28, S29, S30, and S37) harbored a series of amino acid substitutions at specific positions within the protein. Silent changes were also present in some of the isolates (Table 4). Of note, susceptible isolate S8 harbored the same amino acid substitutions as the wild-type isolate used for the cloning of *acrR*, isolate KP3. These findings indicated that the changes represented genetic variation and were not linked to the OST phenotype (Table 4 and Fig. 2). A second group of five isolates

TABLE 4. Amino acid substitutions and mutations identified in AcrR[a]

Strain	DNA change	Amino acid and codon substitution or mutation
S6	7 aa[b] substitutions	P160R, G163A, F171S, R172G, L194V, F196I, K200M
S8, S30, S37	7 aa substitutions, one silent change	P160R, G163A, F171S, R172G, L194V, F196I, K200M, L186L (CTG→CTT)
S28, S29	7 aa substitutions, three silent changes	P160R, G163A, F171S, R172G, L194V, F196I, K200M, E146E (GAA→GAG), L186L (CTG→CTT), L197L (CTG→TTG)
KP3	8 aa substitutions	A20T, P160R, G163A, F171S, R172G, L194V, F196I, K200M
S5	11-bp deletion, frameshift mutation	Δ144–148, TLKE
S10	11-bp deletion, frameshift mutation	Δ144–148, TLKE
S13	11-bp deletion, frameshift mutation	Δ144–148, TLKE
S36	11-bp deletion, frameshift mutation	Δ144–148, TLKE
S7[c]	15-bp deletion	Δ128–132, QAQRQ

[a] In comparison with the *K. pneumoniae* AcrR sequence in the GenBank database (GenBank accession no. AJ318073).
[b] aa, amino acid.
[c] S7 has the same amino acid substitutions as S6.

TABLE 5. Effect of complementation with wild-type AcrR on fluoroquinolone susceptibility

Strain[a]	MIC (μg/ml)[b]	
	Norfloxacin	Ciprofloxacin
S5	>256	64
S5/pTS003*acrR*	96	8
S5/pTS003	128	64
S10	>256	64
S10/pTS003*acrR*	64	8
S10/pTS003	128	64
S13	>256	>128
S13/pTS003*acrR*	256	64
S13/pTS003	>256	>128
S36	>256	>128
S36/pTS003*acrR*	192	64
S36/pTS003	>256	>128
S7	96	8
S7/pTS003*acrR*	64	2
S7/pTS003	64	8

[a] pTS003*acrR* is the vector containing *Klebsiella acrR;* pTS003 contains the vector only.
[b] MIC were determined by E-test (norfloxacin) and doubling agar dilutions (ciprofloxacin).

consisted of four isolates (isolates S5, S10, S13, and S36) with a 4-amino-acid deletion (TLKE; deletion of 11 bp in amino acid positions 144 to 148) that resulted in a frameshift mutation (Table 4). Pulsed-field gel electrophoresis of the isolates in the second subgroup showed they were not clonal (data not shown). A fifth isolate (isolate S7), which produced high levels of AcrA, contained a 5-amino-acid deletion (QAQRQ; deletion of 15 bp in amino acid positions 128 to 132) at positions that differed from the positions at which bases were deleted in the other four isolates (Table 4 and Fig. 2). Isolate S7 harbored the same genetic variation seen in the isolates in the first group with OST (Table 4).

***acrR* complementation.** *Trans*-complementation of strains bearing the mutated *acrR* gene with the wild-type gene resulted in two- to fourfold decreases in the MICs of norfloxacin and ciprofloxacin (Table 5). Decreased AcrA levels were seen by Western blotting and densitometry, indicating that the wild-type *acrR* was able to down-regulate AcrA expression (Fig. 3).

DISCUSSION

In the *K. pneumoniae* clinical isolates studied, mutations associated with fluoroquinolone resistance occurred in both GyrA and ParC at positions identified previously (5, 6, 11, 19, 21, 23). Mutations in *gyrA* were observed in isolates for which the ciprofloxacin MIC was 0.5 μg/ml and greater, and isolates for which the MIC was ≥16 μg/ml were found to have an additional mutation within *parC*. Unlike *E. coli*, *Klebsiella* does not appear to require the presence of double mutations in both *gyrA* and *parC* for higher levels of fluoroquinolone resistance. Three mutations appeared to be sufficient for high-level resistance (Table 3). While the possible involvement of other genes, namely, *gyrB* and *parE*, was not investigated, the role of mutations in these genes in mediating high-level fluoroquinolone resistance has not been described among the other members of the family *Enterobacteriaceae* (34).

Like others, our studies show that the topoisomerase mutations alone were not able to explain the wide range of fluoroquinolone susceptibilities observed for clinically resistant isolates (25, 26). Studies with clinical *E. coli* isolates have shown that increased levels of *marA* expression (25, 35) are associated with increased levels of fluoroquinolone resistance. None of the *Klebsiella* isolates evaluated in this study harbored a mutation within *marR*, and none exhibited *marA* overexpression.

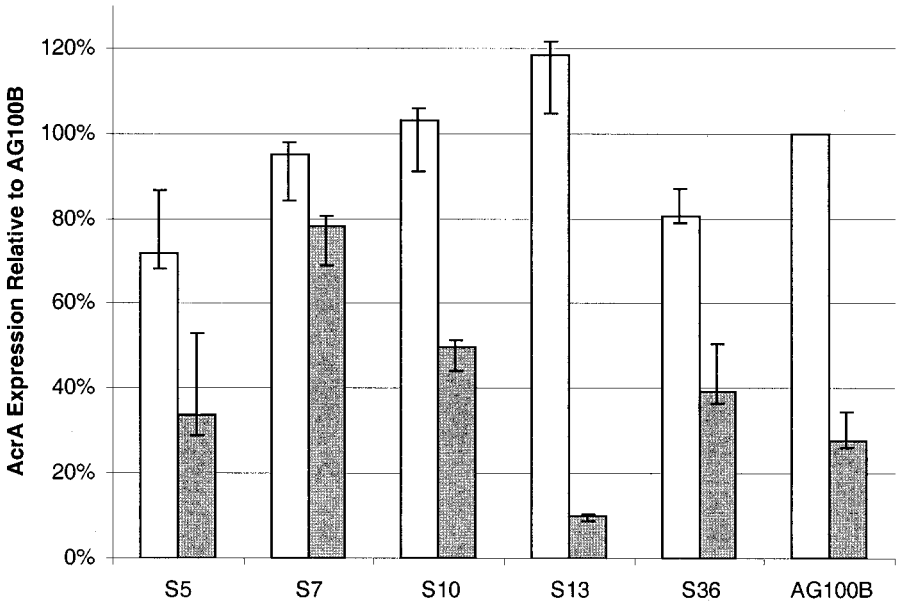

FIG. 3. Effect of wild-type *acrR* on expression of AcrA. White bars, AcrA expression in *acrR* mutant clinical *K. pneumoniae* isolates; gray bars, strains complemented with wild-type *Klebsiella acrR* (pTS003*acrR*). *E. coli* AG100B (*acrR* mutant) served as a positive control.

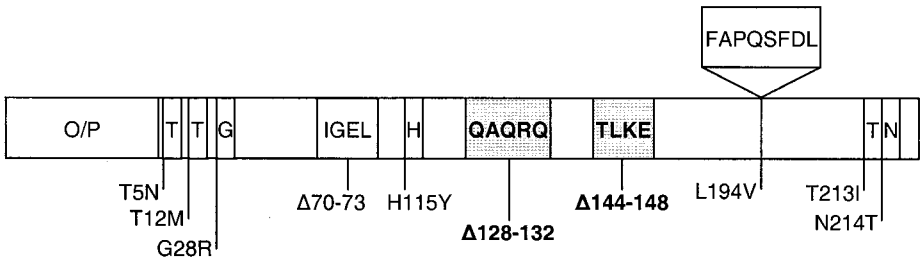

FIG. 4. Functional mutations identified within AcrR from *K. pneumoniae* and *E. coli.* O/P, operator and promoter region of the *acrAB* pump. *E. coli* AcrR mutations are shown on a white background (data are from reference 34). *K. pneumoniae* AcrR mutations are shown on a gray background.

Similarly, no *soxS* overexpression was detected by Northern blotting analysis. Interestingly, increased levels of expression of the related *Klebsiella* transcriptional regulator *ramA* were seen in 3 of the 11 isolates studied. All three isolates also overexpressed AcrA, the membrane fusion protein of the AcrAB-TolC efflux pump. The *ramA* operator and promoter sequences of the three *ramA*-overexpressing strains did not show any changes within this region, suggesting that *ramA* overexpression is mediated by a different locus. An increased level of transcription of *acrA* follows increased levels of expression of regulatory genes *marA* and *soxS*, even in the presence of a functioning AcrR. Heterologous expression of *ramA* in *E. coli* conferred a multidrug resistance phenotype which was dependent on a functional AcrAB pump (T. Schneiders and S. B. Levy, unpublished data). These findings suggest that the overexpression of AcrA is linked to the increased level of transcription of *ramA*. One of the strains which overexpressed *ramA* (strain S7) also harbored a deletion within AcrR, so the role of *ramA* in fluoroquinolone resistance in this isolate is unclear.

Western blotting analysis of AcrA showed overexpression of AcrA which was associated with *acrR* mutations in some of the isolates. Five isolates harbored the same 7-amino-acid substitutions with or without silent changes in AcrR not related to altered levels of AcrA expression (Table 4). This finding indicates that genotypic variation exists between geographically different clinical isolates and is consistent with the recent finding of at least a 5% amino acid variation between the *acrA* GenBank entry (A. Domenech-Sanchez et al., Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-2018, p. 104, 2000) and ciprofloxacin-resistant *Klebsiella* isolates recovered in Italy (23).

Five strains with OST showed *acrR* deletions (Fig. 4). These mutations are different from those described in the *E. coli* AcrR (34) (Fig. 4). A conserved cluster was consistently deleted (TLKE; deletion of 11 bp in amino acid positions 144 to 148), albeit in only four of the clinical isolates; but the strains were not confined to one hospital in Singapore, nor were they found to be clonally related, as determined by pulsed-field gel electrophoresis (data not shown). Finally, the *acrR* deletions described here do not confer a particular level of resistance to ciprofloxacin, as the MICs for these isolates ranged from 16 to 128 μg/ml. Of particular interest is high-level fluoroquinolone-resistant (128 μg/ml) and organic solvent-tolerant isolate S6, which harbored three topoisomerase mutations (two in *gyrA* DNA and one in *parC* DNA) and produced increased levels of AcrA but which had no mutations within the repressor gene (AcrR) and did not have increased levels of expression of any of the regulatory genes investigated. It is likely that other regulatory genes are involved. Complementation with the plasmid containing wild-type *acrR* resulted in decreases in the MICs of both ciprofloxacin and norfloxacin for all *acrR* deletion mutants tested (Table 4). This study and other published data clearly establish a role for efflux pumps such as AcrAB in clinical fluoroquinolone resistance. However, the trend appears to favor the selection of topoisomerase (*gyrA*) mutations prior to the selection of those contributing to efflux pump overexpression. The results presented here support the role of *acrR* mutations and the regulatory locus *ramA* in mediating AcrA overexpression and fluoroquinolone resistance in *K. pneumoniae*.

ACKNOWLEDGMENTS

We thank R. Lin, Kandang, Kerbau Hospital, for collecting the clinical *K. pneumoniae* isolates.

This work was supported by grants from the National Institutes of Health (grant GM 51661/AI 56021) and the Scottish Office Department of Health (grant K/MRS/50/C2698).

REFERENCES

1. **Alekshun, M. N., and S. B. Levy.** 1999. Alteration of the repressor activity of MarR, the negative regulator of the *Escherichia coli marRAB* locus, by multiple chemicals in vitro. J. Bacteriol. **181:**4669–4672.
2. **Brisse, S., D. Milatovic, A. C. Fluit, J. Verhoef, N. Martin, S. Scheuring, K. Kohrer, and F. J. Schmitz.** 1999. Comparative in vitro activities of ciprofloxacin, clinafloxacin, gatifloxacin, levofloxacin, moxifloxacin, and trovafloxacin against *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, and *Enterobacter aerogenes* clinical isolates with alterations in GyrA and ParC proteins. Antimicrob. Agents Chemother. **43:**2051–2055.
3. **Brisse, S., D. Milatovic, A. C. Fluit, J. Verhoef, and F. J. Schmitz.** 2000. Epidemiology of quinolone resistance of *Klebsiella pneumoniae* and *Klebsiella oxytoca* in Europe. Eur. J. Clin. Microbiol. Infect. Dis. **19:**64–68.
4. **Chevalier, J., J. M. Pages, A. Eyraud, and M. Mallea.** 2000. Membrane permeability modifications are involved in antibiotic resistance in *Klebsiella pneumoniae.* Biochem. Biophys. Res. Commun. **274:**496–499.
5. **Deguchi, T., A. Fukuoka, M. Yasuda, M. Nakano, S. Ozeki, E. Kanematsu, Y. Nishino, S. Ishihara, Y. Ban, and Y. Kawada.** 1997. Alterations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV in quinolone-resistant clinical isolates of *Klebsiella pneumoniae.* Antimicrob. Agents Chemother. **41:**699–701.
6. **Deguchi, T., T. Kawamura, M. Yasuda, M. Nakano, H. Fukuda, H. Kato, N. Kato, Y. Okano, and Y. Kawada.** 1997. In vivo selection of *Klebsiella pneumoniae* strains with enhanced quinolone resistance during fluoroquinolone treatment of urinary tract infections. Antimicrob. Agents Chemother. **41:**1609–1611.
7. **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.
8. **George, A. M., and S. B. Levy.** 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol. **155:**531–540.
9. **Greenberg, J. T., J. H. Chou, P. A. Monach, and B. Demple.** 1991. Activation

of oxidative stress genes by mutations at the *soxQ*/*cfxB*/*marA* locus of *Escherichia coli.* J. Bacteriol. **173:**4433–4439.
10. **Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli.* Proc. Natl. Acad. Sci. USA **87:**6181–6185.
11. **Hernandez-Alles, S., S. Alberti, D. Alvarez, A. Domenech-Sanchez, L. Martinez-Martinez, J. Gil, J. M. Tomas, and V. J. Benedi.** 1999. Porin expression in clinical isolates of *Klebsiella pneumoniae.* Microbiology **145:**673–679.
12. **Jellen-Ritter, A. S., and W. V. Kern.** 2001. Enhanced expression of the multidrug efflux pumps AcrAB and AcrEF associated with insertion element transposition in *Escherichia coli* mutants selected with a fluoroquinolone. Antimicrob. Agents Chemother. **45:**1467–1472.
13. **Kern, W. V., M. Oethinger, A. S. Jellen-Ritter, and S. B. Levy.** 2000. Non-target gene mutations in the development of fluoroquinolone resistance in *Escherichia coli.* Antimicrob. Agents Chemother. **44:**814–820.
14. **Komatsu, T., M. Ohta, N. Kido, Y. Arakawa, H. Ito, and N. Kato.** 1991. Increased resistance to multiple drugs by introduction of the *Enterobacter cloacae romA* gene into OmpF porin-deficient mutants of *Escherichia coli* K-12. Antimicrob. Agents Chemother. **35:**2155–2158.
15. **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.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
17. **Linde, H. J., F. Notka, C. Irtenkauf, J. Decker, J. Wild, H. H. Niller, P. Heisig, and N. Lehn.** 2002. Increase in MICs of ciprofloxacin in vivo in two closely related clinical isolates of *Enterobacter cloacae.* J. Antimicrob. Chemother. **49:**625–630.
18. **Maneewannakul, K., and S. B. Levy.** 1996. Identification for *mar* mutants among quinolone-resistant clinical isolates of *Escherichia coli.* Antimicrob. Agents Chemother. **40:**1695–1698.
19. **Martinez-Martinez, L., I. Garcia, S. Ballesta, V. J. Benedi, S. Hernandez-Alles, and A. Pascual.** 1998. Energy-dependent accumulation of fluoroquinolones in quinolone-resistant *Klebsiella pneumoniae* strains. Antimicrob. Agents Chemother. **42:**1850–1852.
20. **Martinez-Martinez, L., S. Hernandez-Alles, S. Alberti, J. M. Tomas, V. J. Benedi, and G. A. Jacoby.** 1996. In vivo selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum-cephalosporins. Antimicrob. Agents Chemother. **40:**342–348.
21. **Martinez-Martinez, L., A. Pascual, C. Conejo Mdel, I. Garcia, P. Joyanes, A. Domenech-Sanchez, and V. J. Benedi.** 2002. Energy-dependent accumulation of norfloxacin and porin expression in clinical isolates of *Klebsiella pneumoniae* and relationship to extended-spectrum β-lactamase production. Antimicrob. Agents Chemother. **46:**3926–3932.
22. **Martinez-Martinez, L., A. Pascual, S. Hernandez-Alles, D. Alvarez-Diaz, A. I. Suarez, J. Tran, V. J. Benedi, and G. A. Jacoby.** 1999. Roles of beta-lactamases and porins in activities of carbapenems and cephalosporins against *Klebsiella pneumoniae.* Antimicrob. Agents Chemother. **43:**1669–1673.
23. **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.
24. **Notka, F., H. J. Linde, A. Dankesreiter, H. H. Niller, and N. Lehn.** 2002. A C-terminal 18 amino acid deletion in MarR in a clinical isolate of *Escherichia coli* reduces MarR binding properties and increases the MIC of ciprofloxacin. J. Antimicrob. Chemother. **49:**41–47.
25. **Oethinger, M., W. V. Kern, A. S. Jellen-Ritter, L. M. McMurry, and S. B. Levy.** 2000. Ineffectiveness of topoisomerase mutations in mediating clinically significant fluoroquinolone resistance in *Escherichia coli* in the absence of the AcrAB efflux pump. Antimicrob. Agents Chemother. **44:**10–13.
26. **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.
27. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178:**306–308.
28. **Park, Y. H., J. H. Yoo, D. H. Huh, Y. K. Cho, J. H. Choi, and W. S. Shin.** 1998. Molecular analysis of fluoroquinolone-resistance in *Escherichia coli* on the aspect of gyrase and multiple antibiotic resistance (*mar*) genes. Yonsei Med. J. **39:**534–540.
29. **Paterson, D. L., L. Mulazimoglu, J. M. Casellas, W. C. Ko, H. Goossens, A. Von Gottberg, S. Mohapatra, G. M. Trenholme, K. P. Klugman, J. G. McCormack, and V. L. Yu.** 2000. Epidemiology of ciprofloxacin resistance and its relationship to extended-spectrum beta-lactamase production in *Klebsiella pneumoniae* isolates causing bacteremia. Clin. Infect. Dis. **30:**473–478.
30. **Phillips, I., J. M. Andrews, E. Bridson, E. M. Cooke, H. A. Holt, R. C. Spencer, R. Wise, A. J. Bint, D. F. J. Brown, A. King, and R. J. Williams.** 1991. A guide to sensitivity testing. J. Antimicrob. Chemother. **27:**1–50.
31. **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.
32. **Podschun, R., and U. Ullmann.** 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. **11:**589–603.
33. **Poirel, L., C. Heritier, I. Podglajen, W. Sougakoff, L. Gutmann, and P. Nordmann.** 2003. Emergence in *Klebsiella pneumoniae* of a chromosome-encoded SHV β-lactamase that compromises the efficacy of imipenem. Antimicrob. Agents Chemother. **47:**755–758.
34. **Wang, H., J. L. Dzink-Fox, M. Chen, and S. B. Levy.** 2001. Genetic characterization of highly fluoroquinolone-resistant clinical *Escherichia coli* strains from China: role of *acrR* mutations. Antimicrob. Agents Chemother. **45:**1515–1521.
35. **Webber, M. A., and L. J. Piddock.** 2001. Absence of mutations in *marRAB* or *soxRS* in *acrB*-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of *Escherichia coli.* Antimicrob. Agents Chemother. **45:**1550–1552.
36. **Wu, J., and B. Weiss.** 1992. Two-stage induction of the *soxRS* (superoxide response) regulon of *Escherichia coli.* J. Bacteriol. **174:**3915–3920.