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Klebsiella pneumoniae AcrAB Efflux Pump Contributes to Antimicrobial Resistance and Virulence▽

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Respiratory infections caused by *Klebsiella pneumoniae* are characterized by high rates of mortality and morbidity. Management of these infections is often difficult, due to the high frequency of strains that are resistant to multiple antimicrobial agents. Multidrug efflux pumps play a major role as a mechanism of antimicrobial resistance in Gram-negative pathogens. In the present study, we investigated the role of the *K. pneumoniae* AcrRAB operon in antimicrobial resistance and virulence by using isogenic knockouts deficient in the AcrB component and the AcrR repressor, both derived from the virulent strain 52145R. We demonstrated that the AcrB knockout was more susceptible, not only to quinolones, but also to other antimicrobial agents, including β-lactams, than the wild-type strain and the AcrR knockout. We further showed that the AcrB knockout was more susceptible to antimicrobial agents present in human bronchoalveolar lavage fluid and to human antimicrobial peptides than the wild-type strain and the AcrR knockout. Finally, the AcrB knockout exhibited a reduced capacity to cause pneumonia in a murine model, in contrast to the wild-type strain. The results of this study suggest that, in addition to contributing to the multidrug resistance phenotype, the AcrAB efflux pump may represent a novel virulence factor required for *K. pneumoniae* to resist innate immune defense mechanisms of the lung, thus facilitating the onset of pneumonia.

Klebsiella pneumoniae is one of the most prevalent nosocomial enterobacterial pathogens, causing infections that range from mild urinary infections to severe bacteremia and pneumonia with a high rate of mortality and morbidity (33). Pulmonary infections due to *K. pneumoniae* are often characterized by a rapid progressive clinical course complicated by lung abscesses and multilobular involvement that leaves scant time to establish an effective antibiotic treatment. In addition, an increasing emergence of multidrug resistance among *K. pneumoniae* nosocomial isolates has limited the therapeutic options for treatment of these infections (4).

Fluoroquinolones have been considered an adequate therapeutic option; however, several studies indicate that an increasing percentage of *Klebsiella* strains are resistant to these antimicrobials (11, 21, 41). Fluoroquinolone resistance has been associated with mutations in the quinolone resistance-determining region of the *gyrA* and or *parC* gene (19), coding for the target proteins DNA gyrase and topoisomerase IV, respectively. Plasmid-mediated resistance to quinolones has also been described (23), and its frequency seems to be increasing in recent years (34, 41). Besides topoisomerase mutations and plasmids, altered permeability (usually because of porin loss) and energy-dependent efflux have also been shown to contribute to the fluoroquinolone resistance phenotype in *K. pneumoniae* (21, 22). One of the efflux systems involved in this resistance phenotype is the AcrAB multidrug efflux system that in *K. pneumoniae* is encoded by the *acrRAB* operon. In this operon, *acrR* encodes the AcrAB repressor, while *acrA* and *acrB* encode a periplasmic lipoprotein of 40 kDa, anchored to the inner membrane, that bridges the outer and inner membranes and an integral membrane protein of 113.5 kDa with 12 membrane-spanning α-helices, located in the cytoplasmic membrane, respectively (12). AcrB connects with TolC, an outer membrane protein that belongs to a family of envelope proteins found in all Gram-negative bacteria and that is essential for the expulsion of a plethora of compounds (13).

Studies by Mazzariol et al. and Hasdemir et al. reported a correlation between reduced susceptibility to quinolones and AcrA overexpression in several quinolone-resistant clinical *K. pneumoniae* strains, although the genetic basis of this overexpression was not described (17, 24). Schneiders et al. found that increased AcrAB efflux pump expression in 19 fluoroquinolone-resistant *K. pneumoniae* strains was caused by either mutations in the AcrAB repressor, AcrR, or overexpression of the transcriptional regulator RamA (36). However, little is known about the specific and direct role of the AcrAB multidrug efflux pump in the resistance of *K. pneumoniae* to other antimicrobial agents.

On the other hand, it is known that some bacterial efflux pumps export not only antibiotics and other substances, such as dyes and detergents, but also host-derived antimicrobial agents (32). In addition, there is accumulating evidence that efflux

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TABLE 1. Oligonucleotide primers used in this study

Name	Sequence (5′–3′)	Target gene	Use
AcrU6	GTGGTTAAAACGCTGG	*acrB* internal fragment	*acrB* probe
AcrL3	ATTAATGATGCTCAACCTGATG		
AcrB1	AAGCTTAACTTAAACAGGAGC	*acrB*	Construction of integrant plasmid pQE58
AcrB2	TTCGAAGAAGATTAATGATGCTC		
AcrRF1	GATTCTAGATGTTGCTCTGC	*acrR* internal fragment	*acrR* probe, construction of integrant plasmid pQE61, and RT-PCR amplification of *acrR*
AcrRR1	CGTCGTCTAGAATTGATAC		
AcrU7	GAGCTGATTGACCAGG	*acrB* internal fragment	RT-PCR amplification of *acrB*
AcrL4	CTGATAAGACATCCCGGTCCA		
U681	CGGTTACGGCCAGTGGGAATA	*ompK36* internal fragment	RT-PCR amplification of housekeeping gene *ompK36*
L1316	GACGCAGACCGAAATCGAACT		

pumps that confer clinically relevant antibiotic resistance are important for the pathogenicity of the bacterium (32). Thus, different studies have shown that lack of efflux pump expression by a Gram-negative bacterium has a deleterious effect on the ability of the bacterium to be pathogenic in animal models (5, 6, 18, 20, 27, 39). However, the role, if any, that the AcrAB efflux pump plays in the pathogenesis of *K. pneumoniae* respiratory infections has not been investigated.

In this work, we constructed knockouts in the AcrAB repressor, AcrR, and in the AcrB efflux system component to test the role of the AcrAB efflux pump in resistance to fluoroquinolones and other antimicrobial agents, including human antimicrobial peptides. Finally, we also studied whether the pump plays any role in *K. pneumoniae* pneumonia.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. *K. pneumoniae* strain 52145 is a previously described clinical isolate (serotype 01:K2) (25). A spontaneous rifampin-resistant mutant derived from strain 52145, designated 52145R, was used in this study. The *Escherichia coli* strains used in the cloning experiments were DH5α and strain S17-1 λ*pir*, in which the *pir* gene encodes the protein π, essential for replication of plasmid pFS100 (35). Plasmids pCR2.1 and pGEM-T (Invitrogen) are TA cloning vectors used for cloning PCR products. Plasmid pFS100 was used to create insertion-duplication mutations by homologous recombination (35). Bacterial cells were grown in Luria Bertani (LB) broth at 37°C with shaking or solidified with 1.5% agar. When necessary, media were supplemented with kanamycin (50 μg/ml) (Sigma-Aldrich, Madrid, Spain).

DNA procedures. Plasmid DNA was isolated using the Wizard Miniprep kit (Promega, Madrid, Spain) according to the manufacturer's instructions. Isolation of genomic DNA, PCRs, transformations, and conjugations were carried out by standard techniques (2). T4 DNA ligase and restriction endonucleases were used following the manufacturer's recommendations (GE Healthcare, Barcelona, Spain). DNA fragments prepared by restriction enzyme digestion were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. For Southern blot analysis and probe labeling and detection, we used the ECL kit (GE Healthcare, Barcelona, Spain) according to the manufacturer's protocol. The probes for *acrB* and *acrR* were obtained by PCR amplification of *K. pneumoniae* 52145R genomic DNA with primers AcrU6/AcrL3 and AcrRF1/AcrRR1, respectively (Table 1).

Generation of *acrB* and *acrR* knockouts from *K. pneumoniae* strain 52145R. To construct an *acrB* knockout from *K. pneumoniae* strain 52145R, the entire *acrB* gene obtained by PCR amplification with primers AcrB1 and AcrB2 (Table 1) was cloned into pCR2.1 to give pQE57. A KpnI internal *acrB* 800-bp fragment from pQE57 was cloned into the π protein-dependent shuttle vector pFS100 to give plasmid pQE58. To generate an *acrR* knockout, an internal fragment of the *acrR* gene was amplified by PCR from *K. pneumoniae* 52145R genomic DNA with primers AcrRF1/AcrRR1. The PCR product was cloned in pGEM-T to give pQE60. An EcoRI fragment from pQE60 containing the internal fragment of *acrR* was cloned in the shuttle vector pFS100 digested with EcoRI to give plasmid pQE61. Plasmids pQE58 and pQE61 were introduced into *K. pneumoniae* by conjugation. To select integrants of plasmid pQE58 or pQE61 into the *K. pneumoniae* chromosome, thereby disrupting expression of *acrB* or *acrR*, respectively, an aliquot of each conjugation was spread on rifampin-kanamycin-containing agar. One integrant from each conjugation, designated *K. pneumoniae* 52145ΔacrB or *K. pneumoniae* 52145ΔacrR, was further investigated.

RNA extraction and RT-PCR. Total cellular RNA was isolated from *K. pneumoniae* using the Qiagen Rneasy Mini Kit (Qiagen, Barcelona, Spain) according to the manufacturer's instructions. *acrB*, *acrR*, and *ompK36* transcripts were detected by reverse transcription (RT)-PCR using primers AcrU7/AcrL4, AcrRF1/AcrRR1, and U681/L1316, respectively (Table 1). RT-PCR was performed using the Access RT-PCR system (Promega, Madrid, Spain) by a 45-min reaction at 48°C using 15 ng of total cellular RNA as a template, followed by a 5-min hot start and 20 to 30 cycles with the following reaction conditions: 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min. The RT-PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Determination of CPS and LPS expression. Capsule polysaccharide (CPS) and lipopolysaccharide (LPS) expression was quantified by an inhibition enzyme-linked immunosorbent assay (iELISA). For this purpose, plates were coated with 1 μg of either purified CPS type 2 or LPS O side chain type 1 per well. After a blocking step with phosphate-buffered saline (PBS), pH 7.4, containing 1% bovine serum albumin (BSA), the plates were incubated with serial dilutions of CPS extracts or LPS extracts and antisera against CPS type 2 or LPS O side chain type 1, respectively. Bound antibodies were detected with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G and developed with *p*-nitrophenyl phosphate. Incubations with antisera diluted in PBS-1% BSA were carried out at 37°C for 1 h and were always followed by PBS washes. Known amounts of CPS and LPS purified by the methods of Wilkinson and Sutherland (43) and Westphal and Jann (42) were used to construct a standard curve.

Susceptibility testing. The MICs of antimicrobial agents were determined using Etest strips (AB Biodisk, Solna, Sweden) following the manufacturer's instructions.

Bacterial survival experiments. For the bronchoalveolar lavage fluid (BALF) bactericidal assays, BALF from healthy subjects was collected as previously described (8). This procedure was approved by the local ethics committee, and written informed consent was obtained before collection of the human samples. The BALF (120 to 200 ml from each subject) was centrifuged to eliminate the cells, and a protease cocktail inhibitor (Sigma-Aldrich, Madrid, Spain) was added to the supernatants. After lyophilization, the BALF was resuspended in sterile water to produce a 200-fold-concentrated solution with respect to the initial BALF volume. BALFs obtained from three different subjects were pooled before the antimicrobial assay described below was performed. For the bactericidal assays using BALF, 10^3 bacterial cells grown to the exponential phase of growth and resuspended in 150 μl of PBS were mixed with 50 μl of BALF. After 1 h of incubation at 37°C, viable bacteria were counted by plating appropriate dilutions on LB agar plates. The BALF bactericidal effect was calculated as the survival percentage, with the bacterial counts obtained at the beginning of the experiment set to 100%.

To test the effect of polymyxin B (Sigma-Aldrich, Madrid, Spain), *K. pneumoniae* strains were grown at 37°C in 5 ml of LB medium and were harvested (5,000 × *g*; 15 min; 5°C) in the exponential phase of growth, and a suspension containing ca. 2.1×10^5 CFU/ml was prepared in 1% (wt/vol) tryptone-PBS (pH 7.4). Then, 10 μl of this cell suspension was mixed in Eppendorf tubes with polymyxin B (0.1 μg/ml) in a volume of 200 μl, followed by incubation at 37°C for 30 min. In parallel experiments, cells were de-energized with 50 μM carbonyl-cyanide-*m*-chlorophenylhydrazone (CCCP). In order to evaluate the effect of reenergization of cells with glucose, the relative survival was also determined in bacterial suspensions treated with polymyxin B, exposed or not to CCCP, and

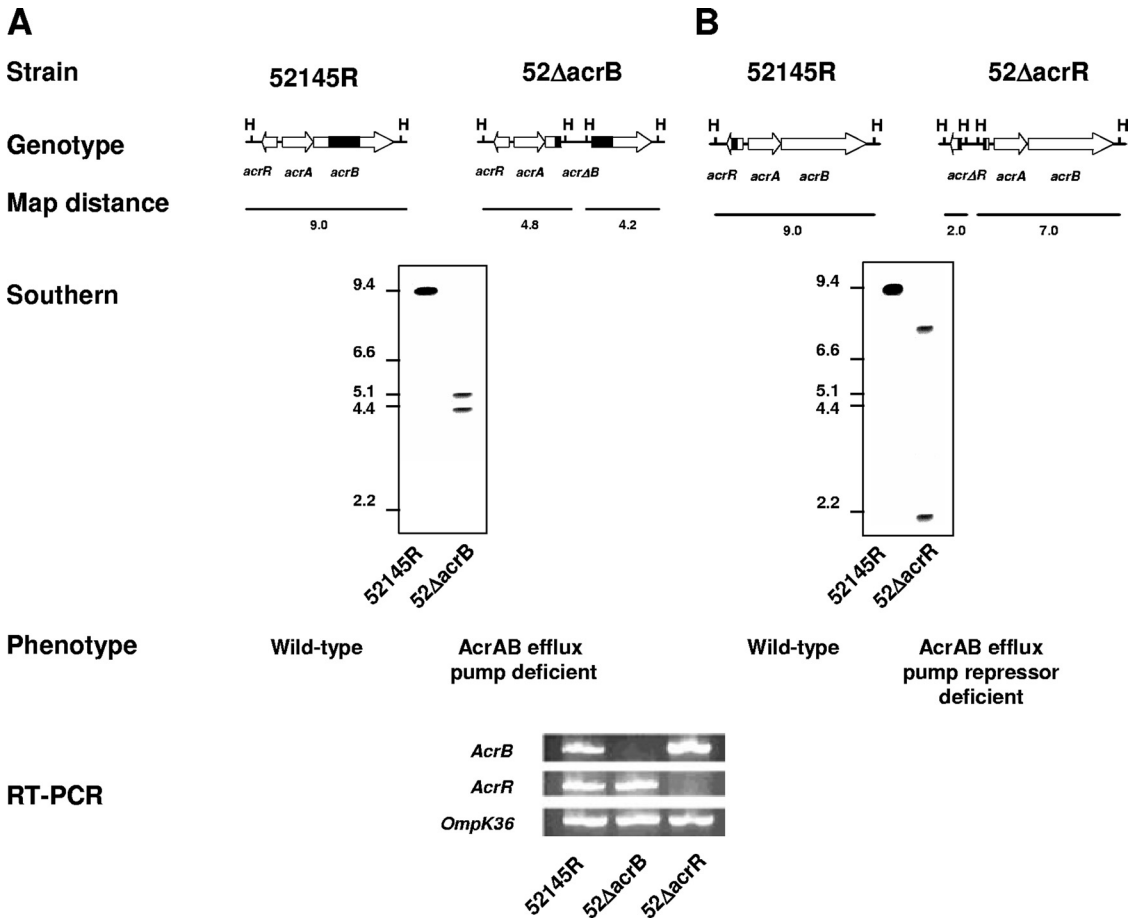

FIG. 1. Schematic representation of the *K. pneumoniae* chromosome in the virulent strain 52145R and its isogenic knockouts 52ΔacrB (A) and 52ΔacrR (B). DNA fragment sizes in the schematic are not to scale. The black boxes represent the probes used in the Southern blot analysis. The lines between the black boxes in the knockout genomes represent the DNA plasmids integrated into the chromosome. H indicates HindIII. The expected sizes of the fragments that hybridize with the probes described above are indicated in kilobases. Southern blot analysis of *K. pneumoniae* wild-type and isogenic knockout chromosomes digested with HindIII is shown. Molecular size markers (in kilobases) are shown to the left of the blots. The expression of the mutated gene in each strain was determined by RT-PCR. For each strain, the product of RT-PCR using primers specific for *acrB*, *acrR*, or *ompK36*, a control gene, is shown.

treated with glucose (0.4%). Colony counts were determined, and the results were expressed as percentages of the colony counts of bacteria not exposed to antibacterial agents.

To test the effect of human neutrophil defensin 1 (HNP-1) (Sigma-Aldrich, Madrid, Spain), and human β-defensin 1 (HBD-1) and HBD-2 (Preprotech, Rocky Hill, NJ), a bacterial suspension containing 10^5 CFU/ml was prepared in 10 mM PBS (pH 6.5)-0.1% tryptic soy broth (TSB)-100 mM NaCl. Next, 5 μl of this suspension was mixed in Eppendorf tubes with the corresponding defensin to get a final volume of 25 μl. After 1 h of incubation, the contents of the Eppendorf tubes were plated on LB agar plates. Colony counts were determined, and the results were expressed as described above.

All bacterial survival experiments were done with duplicate samples on at least three independent occasions.

Murine model of pneumonia. Male (16- to 20-g) ICR-CD1 mice (Harlan Ibérica) were anesthetized and intubated intratracheally using a blunt-end feeding needle (9). Approximately 10^3 CFU of *K. pneumoniae* from an early-log-phase broth culture was suspended in 50 μl of sterile saline solution and inoculated through the blunt-end needle. At 24 h, 48 h, and 72 h, animals ($n = 10$ per group) were sacrificed, and the lungs were aseptically removed and homogenized for quantitative bacterial cultures. All animal experiments were done according to institutional and national guidelines and approved by the Experimental Animal Committee of the institution.

Statistical methods. Comparisons among groups were made by the two-sample *t* test or, when the requirements were not met, by the Mann-Whitney U test. A *P* value of <0.05 was considered statistically significant.

RESULTS

Genotypic and phenotypic characterization of the *K. pneumoniae* AcrRAB operon knockouts. To investigate the role of the AcrAB efflux pump in *K. pneumoniae* antimicrobial resistance and virulence, we constructed two isogenic knockouts from the wild-type strain 52145R. This strain was selected due to its high virulence in a murine model of pneumonia (9). We used insertion-duplication mutagenesis to interrupt *acrB*, required for the synthesis of a functional efflux pump, or *acrR*, required for the synthesis of the repressor of the expression of the efflux pump. A schematic representation of the wild-type 52145R and the knockout 52ΔacrB and 52ΔacrR chromosomes is shown in Fig. 1. Southern blot analysis of the genomic DNA of the knockouts using specific probes for *acrB* or *acrR* confirmed that two incomplete copies of the genes were generated by the integration of the plasmid. RT-PCR analysis confirmed that the mutations abolished the transcription of *acrB* or *acrR*. In both knockout strains, *ompK36*, a porin-coding gene used as a control, was transcribed efficiently.

TABLE 2. Antimicrobial susceptibility (MICs) of *K. pneumoniae* 52145R and its derived isogenic knockouts, 52ΔacrB and 52ΔacrR

Antimicrobial agent	MIC[a]		
	52145R	52ΔacrB	52ΔacrR
Nalidixic acid	4	0.5	8
Ciprofloxacin	0.06	0.008	0.12
Levofloxacin	0.09	0.006	0.19
Imipenem	0.125	0.125	0.125
Ertapenem	0.012	0.012	0.012
Meropenem	0.03	0.03	0.03
Cefoxitin	8	4	32
Cefotaxime	0.125	0.06	4
Erythromycin	32	8	32
Tetracycline	2	0.25	4
Chloramphenicol	2	1	4
Tobramycin	0.25	0.125	0.25
Gentamicin	0.25	0.06	0.25

[a] MICs are expressed in μg/ml.

Role of the AcrAB efflux pump in *K. pneumoniae* antimicrobial resistance. The susceptibilities of the wild-type *K. pneumoniae* strain 52145R and its derived isogenic efflux pump knockouts, 52ΔacrB and 52ΔacrR, to a number of antibiotics were determined (Table 2). It is noteworthy that the MICs of most of the antibiotics tested in strain 52145R were low. However, we observed differences in the MICs of various antibiotics between the wild-type strain and the knockouts. As expected, the MICs of all quinolones were lower for 52ΔacrB than for the wild type, whereas a slight increase in the MICs was observed for 52ΔacrR. The same trend was observed for β-lactams. MICs were lower for 52ΔacrB and higher for 52ΔacrR than for the wild-type strain. The MICs of carbapenems were similar for all strains. The MIC of erythromycin was dramatically reduced in the *acrB* knockout, while there were modest decreases in the MICs of tetracycline, chloramphenicol, and the aminoglycosides.

Altogether, these results confirm the reliability of our knockouts and indicate that the AcrAB efflux pump is involved in the expulsion of different antimicrobial agents.

The role of the AcrAB efflux pump in *K. pneumoniae* pneumonia. We explored whether the AcrAB efflux pump plays any role in resistance against the antibacterial agents found in the BALF. The efflux pump knockout 52ΔacrB was more susceptible to BALF than the wild-type strain 52145R. One hour of incubation in the presence of BALF was enough to kill the knockout 52ΔacrB (5% ± 3% survival), whereas the survival rates of the parent strain 52145R and the knockout 52ΔacrR were less affected (53% ± 5% and 62% ± 7% survival, respectively). Given that antimicrobial peptides are among the array of antibacterial compounds present in BALF, we hypothesized that 52ΔacrB could be more susceptible to these agents than 52145R. Thus, we tested the sensitivities of 52145R and the knockouts to different antimicrobial agents (Fig. 2). Polymyxin B is considered a model of antimicrobial-peptide action (16), whereas HNP-1, HBD-1, and HBD-2 are three antimicrobial peptides reported to be present in human BALF (1, 15). Confirming our hypothesis, 52ΔacrB was significantly more susceptible to polymyxin B, HNP-1, HBD-1, and HBD-2 (Fig. 2) ($P < 0.05$) than 52145R. These differences were not due to reduced expression of CPS and LPS, molecules involved in resistance to antimicrobial peptides in *K. pneumoniae* (8), because 52145R

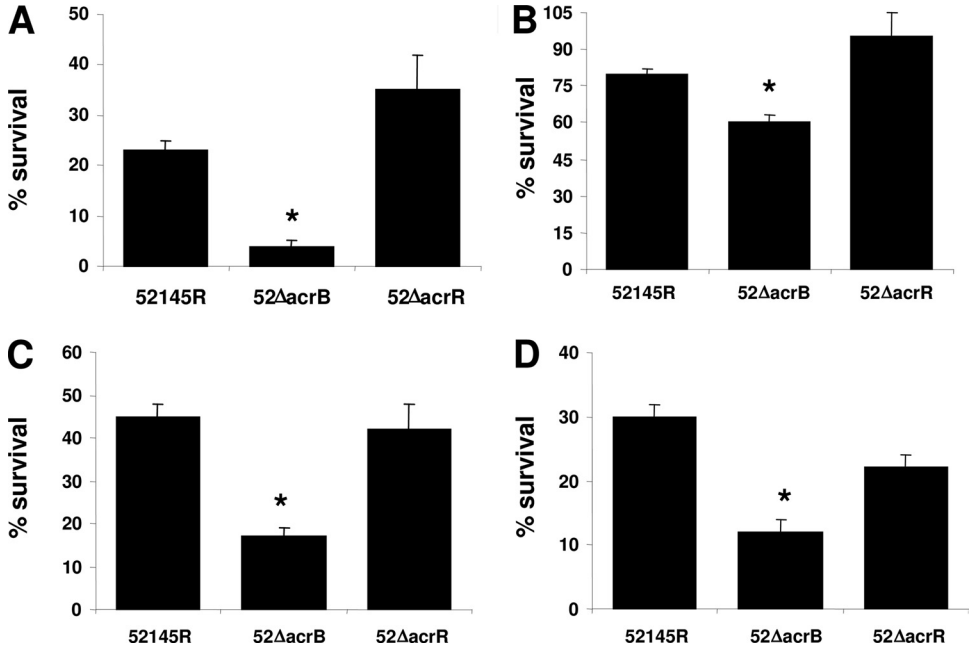

FIG. 2. Resistance of *K. pneumoniae* 52145R and the derived knockouts 52ΔacrB and 52ΔacrR to polymyxin B and human antimicrobial peptides. Bacterial cells were incubated with polymyxin B (0.1 μg/ml) (A), HNP-1 (30 μg/ml) (B), β-defensin 1 (0.1 μg/ml), or β-defensin 2 (0.1 μg/ml). The survival of bacteria (percentage of colony counts of cells not exposed to the agents) is shown. Each point represents the mean and standard deviation of at least three independent experiments run in duplicate. Significant survival differences between the strains ($P < 0.05$) are indicated by asterisks.

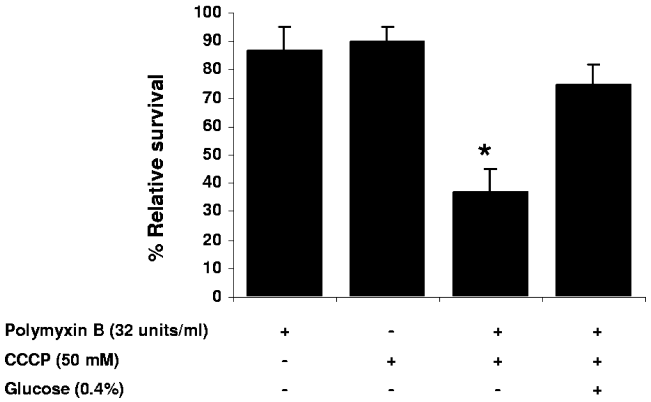

FIG. 3. Effect of polymyxin B on *K. pneumoniae* 52145R. Bacterial cells were exposed to polymyxin B (0.1 μg/ml) alone or in the presence of glucose (0.4%), CCCP (50 μM), and CCCP plus glucose. The survival of bacteria is expressed as the percentage of the colony counts of cells not exposed to polymyxin B after 1 h of incubation. The error bars display the standard deviations from the mean of three experiments, each one run in duplicate. Significant survival differences ($P < 0.05$) are indicated by the asterisk.

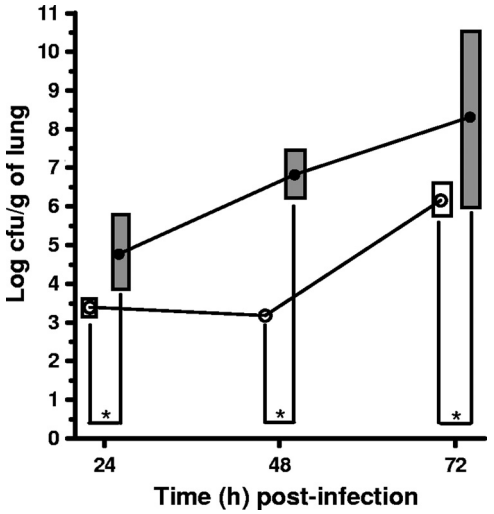

FIG. 4. Effect of AcrAB efflux pump deficiency on *K. pneumoniae* lung infection in vivo. Mice ($n = 10$ per group) were intratracheally inoculated with the wild-type strain 52145R (gray bars) or the AcrB-deficient strain 52ΔacrB (white bars). The number of bacterial cells in lung homogenates was determined at 24, 48, and 72 h postinfection. The results that are significantly different from those in untreated controls are denoted by asterisks.

and 52ΔacrB expressed similar amounts of both polysaccharides (data not shown). The results also showed that 52ΔacrR was slightly more resistant to polymyxin B and HNP-1 than the wild type, although the differences were not statistically significant.

To further confirm the role of the energy-driven efflux pump of antimicrobial peptides, we asked whether dissipation of the proton-motive force increases susceptibility to polymyxin B. An uncoupler, CCCP, was used to interfere with proton-motive force-dependent efflux pumps. CCCP greatly reduced the survival of 52145R against polymyxin B (Fig. 3), indicating that polymyxin B was pumped out by a mechanism that required energy. In turn, the addition of glucose to CCCP-treated cells increased the relative survival rate to levels similar to those observed in untreated bacteria (Fig. 3).

Finally we tested the ability of the knockout 52ΔacrB to cause pneumonia in a mouse model. Mice were infected intratracheally with 52145R or 52ΔacrB, and the bacterial loads in lung homogenates were determined at days 1, 2, and 3 postinfection. As shown in Fig. 4, lungs from mice infected with the *acrB* knockout presented significantly lower (asterisks in Fig. 4) ($P < 0.05$) bacterial loads than those infected with the wild-type strain.

DISCUSSION

Sequencing analysis of the genomes of a wide variety of bacterial species has indicated that most of them encode several efflux pump systems. *K. pneumoniae* encodes an AcrAB system homologous to the system described in *E. coli.* In addition, only a few similar multidrug efflux systems have been characterized in *Klebsiella* spp. (14, 28, 10), although the sequencing data for *K. pneumoniae* strain MGH78578 indicate that many others potentially are present. In general, most of the clinical studies have focused on the roles of these efflux pumps in antibiotic resistance. However, it is reasonable to postulate that the function of these efflux pumps within the human body is the export of toxic compounds present in their natural environment, such as bile salts, fatty acids, and hormones. Furthermore, few studies have studied the contributions of these efflux pumps to bacterial virulence.

In this study, we have characterized the contribution of the *K. pneumoniae* AcrAB efflux pump in resistance to quinolones and other antibiotics by using *acrB* and *acrR* knockouts derived from the virulent strain 52145R. *acrB* deficiency resulted in an increased susceptibility to quinolones, which is consistent with previous reports showing that overexpression of AcrB due to mutations in *acrR* caused resistance to ciprofloxacin in several *K. pneumoniae* clinical isolates (17, 24, 36). Moreover, we observed a decrease in the MICs of erythromycin, tetracycline, chloramphenicol, amynoglycosides, and β-lactams, except carbapenems, for 52145ΔacrB in comparison with those for the wild-type strain, indicating that this efflux system may recognize multiple substrates, as already noted for the homologous proteins from *Enterobacter cloacae* and *E. coli* (26, 31). This fact suggests that this efflux pump system, in combination with extended-spectrum β-lactamases and porin deficiency, may significantly contribute to the multidrug resistance phenotype observed in some epidemic *K. pneumoniae* strains and supports the idea that the involvement of efflux pumps in ß-lactam resistance is especially underestimated in clinical isolates (29).

The *acrR* knockout showed a moderate increase in the MICs of most of the antimicrobial agents tested compared to the parent strain, 52145R. In contrast with previous studies, where mutations in the repressor led to a marked increase in resistance to quinolones due to overexpression of AcrAB (24, 36), in this work, AcrR deficiency did not increase the MICs of quinolones dramatically. There are two nonexclusive hypotheses to account for this result. First, there must be other factors, such as RamA, involved in the regulation of the expression of this efflux pump in this strain. Second, other

mechanisms of resistance (i.e., other efflux pumps) can be required for the strain to become resistant to quinolones.

In this study, we have presented evidence showing that the *K. pneumoniae* AcrAB system also mediates resistance against antimicrobial peptides reported to be present in the lung. Thus, HBD-1 and HBD-2 are synthesized by airway epithelial cells, whereas HNP-1 is released by neutrophils recruited to the site of infection. Furthermore, the concentrations tested were within the range reported to be present in the respiratory fluid of healthy subjects (15), giving physiological relevance to our work. Interestingly, the AcrAB homologue of the plant pathogen *Erwinia amylovora* mediates resistance to plant-derived antibacterial toxins, such as flavonoids, isoprenoids, and alkaloids (7).

Until now, few efflux pumps have been shown to mediate resistance against antimicrobial peptides, the best-characterized examples being the MtrC-MtrD-MtrE efflux pump from *Neisseria gonorrhoeae* and *Neisseria meningitidis*, the MexAB-OprM efflux pump from *Pseudomonas aeruginosa*, and the efflux pump/potassium antiporter from *Yersinia enterocolitica* (37, 40, 30, 3). Therefore, it is tempting to postulate that one of the primary roles of efflux pumps is to pump out antimicrobial agents present in the host tissues. Not surprisingly, antimicrobial peptides are considered one of the first barriers against infections of the innate immune system.

In vivo experiments revealed lower bacterial loads in the lungs of mice infected with 52ΔacrB than in those infected with the wild-type strain. Although significant, these differences were not dramatic, perhaps because other virulence factors, such as CPS or LPS (9, 38), that play major roles in the outcome of pneumonia masked the effects. However, this efflux system may play a crucial role in bacterial resistance against early host defense mechanisms in those strains overexpressing AcrAB selected in vivo during therapy with quinolones. Thus, the association between overexpression of the AcrAB efflux system and resistance to the antimicrobial peptides may raise a serious concern about the use of some antimicrobial agents for the treatment of pneumonia in immunocompromised patients.

In summary, in this article we described the use of two specific knockouts, 52ΔacrB and 52ΔacrR, to test the effects of the AcrAB efflux system on antimicrobial resistance and virulence. We demonstrated that the system is involved in resistance not only to quinolones, but also to other antimicrobial agents. We further showed a novel role for the *K. pneumoniae* AcrAB system: resistance to host antimicrobial peptides. The finding of this novel function stimulates the idea of developing and seeking inhibitors affecting the efflux systems.

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