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Cloning, Nucleotide Sequencing, and Analysis of the AcrAB-TolC Efflux Pump of *Enterobacter cloacae* and Determination of Its Involvement in Antibiotic Resistance in a Clinical Isolate▽

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***Enterobacter cloacae* is an emerging clinical pathogen that may be responsible for nosocomial infections. Management of these infections is often difficult, owing to the high frequency of strains that are resistant to disinfectants and antimicrobial agents in the clinical setting. Multidrug efflux pumps, especially those belonging to the resistance-nodulation-division family, play a major role as a mechanism of antimicrobial resistance in gram-negative pathogens. In the present study, we cloned and sequenced the genes encoding an AcrAcB-TolC-like efflux pump from an *E. cloacae* clinical isolate (isolate EcDC64) showing a broad antibiotic resistance profile. Sequence analysis showed that the *acrR*, *acrA*, *acrB*, and *tolC* genes encode proteins that display 79.8%, 84%, 88%, and 82% amino acid identities with the respective homologues of *Enterobacter aerogenes* and are arranged in a similar pattern. Deletion of the *acrA* gene to yield an AcrA-deficient EcDC64 mutant (EcΔacrA) showed the involvement of AcrAB-TolC in multidrug resistance in *E. cloacae*. However, experiments with an efflux pump inhibitor suggested that additional efflux systems also play a role in antibiotic resistance. Investigation of several unrelated isolates of *E. cloacae* by PCR analysis revealed that the AcrAB system is apparently ubiquitous in this species.**

Multidrug efflux pumps, especially those belonging to the resistance-nodulation-division family, play a major role in establishing the "intrinsic or acquired" resistance of gram-negative bacteria to a wide range of toxic compounds, including antibiotics (20).

A well-studied example is the AcrAB-TolC multidrug resistance (MDR) tripartite pump system of *Escherichia coli*, which confers resistance to a wide variety of lipophilic and amphiphilic compounds, including dyes, detergents, and antimicrobial agents such as ethidium bromide, crystal violet, sodium dodecyl sulfate, bile acids, tetracycline, chloramphenicol, fluoroquinolones, β-lactams, erythromycin, and fusidic acid (17, 18). The presence of similar systems has been reported for other members of *Enterobacteriaceae* (2, 9, 11, 12, 14, 15, 16, 21, 28).

The species of *Enterobacter* that most commonly cause nosocomial infections are *E. cloacae* and *E. aerogenes* (24). The existence of an *acrB*-like gene (11) has previously been identified in *E. cloacae*, although none of the components of the efflux pump have been cloned.

The objectives of the present study were (i) to characterize the genes encoding the AcrAB-TolC-like efflux pump of *E. cloacae* and (ii) to determine the involvement of this efflux pump in MDR in *E. cloacae*. The clinical strain used in the study was an MDR strain of *E. cloacae* (EcDC64) which overexpressed the AcrAB system and also overexpressed the chromosomal *ampC* gene and exhibited a drastic reduction of *ompC* gene expression.

(These results were presented in part at the 45th Interscience Conference on Antimicrobial Agents and Chemotherapy [2a].)

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains used in the study are listed in Table 1. *Enterobacter cloacae* clinical isolate EcDC64 was isolated from a patient admitted to the intensive care unit of the Juan Canalejo Hospital (La Coruña, NW Spain). The bacterial isolate was identified by a MicroScan Walkaway (Dade Behring, Barcelona, Spain), and the identification was confirmed by API 20E (bioMérieux, Marcy l'Etoile, France). *Escherichia coli* TG1 strain was used for cloning procedures. All strains used in the study were maintained at −80°C in 15% (vol/vol) glycerol for cryoprotection until use. The strains were grown on MacConkey agar plates (Becton, Dickinson and Company, NJ), in Luria-Bertani (LB) broth, or on LB agar in the presence of 25 μg of kanamycin/ml or 20 μg of chloramphenicol/ml when required.

Susceptibility testing. Antibiotic susceptibility was determined by the following three different methods, depending on the antimicrobial availability.

The MICs of the following antibiotics were determined by Etest (AB Biodisk, Solna, Sweden) following the manufacturer's criteria: ampicillin, penicillin, amoxicillin-clavulanic acid, piperacillin, cephalothin, cefoxitin, cefuroxime, ceftazidime, aztreonam, cefotaxime, imipenem, meropenem, oxacillin, erythromycin, clindamycin, tetracycline, chloramphenicol, tobramycin, gentamicin, amikacin, ciprofloxacin, tigecycline, linezolid, and trimethoprim-sulfamethoxazole.

Susceptibility to the following antibiotics were determined by the standard disk diffusion method (Becton, Dickinson and Company, NJ) on Mueller-Hinton

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TABLE 1. Bacterial strains and plasmids used in the present study

Strain or plasmid	Features (resistance marker[a])	Source or reference
Strains		
EcDC64	Multiresistant *E. cloacae* clinical strain isolated from a patient admitted to our hospital	This study
EcΔacrA	EcDC64 with *acrA* gene knockout	This study
EcΔacrA(pAP-2)	EcΔAcrA transformed with pAP-2	This study
EcΔacrA(pACYC184)	EcΔAcrA transformed with pACYC184	This study
EcDC64(pAP-3)	EcDC64 transformed with pAP-3	This study
EcDC64(pBGS18)	EcDC64 transformed with pBGS18	This study
E. cloacae Jc 194	Carbapenem-susceptible strain used as reference strain in RT-PCR experiments	This study
Plasmids		
pBGS18	Cloning vector (kanamycin)	26
pACYC184	Cloning vector (chloramphenicol and tetracycline)	3, 22
pKOBEG	Cloning vector (chloramphenicol)	4
pAP-1 (pBGS18-*ampD*)	a*mpD* gene from *E. coli* cloned into pBGS18 (kanamycin)	This study
pAP-2 (pACYC184-*acrA*)	*acrA* gene from EcDC64 isolate cloned into pACYC184 (chloramphenicol)	This study
pAP-3 (pBGS18-*acrR*)	*acrR* gene from *E. aerogenes* cloned into pBGS18 (kanamycin)	This study

[a] Resistance markers for plasmids are shown in parentheses.

agar: telithromycin, fusidic acid, novobiocin, nalidixic acid, rifampin, and norfloxacin.

The broth microdilution method (19) was used for detergents and dyes, sodium dodecyl sulfate, sodium cholate, sodium deoxycholate, acriflavine (Sigma-Aldrich, St. Louis, MO), ethidium bromide, Triton X-100 (AppliChem GmbH, Darmstadt, Germany), and crystal violet (Merck, Darmstadt, Germany). The inoculum of *E. cloacae* cultures consisted of 5×10^5 CFU/ml, and the plates were incubated for 16 to 20 h at 37°C. As the end point, the lowest concentration of the compound that completely inhibited growth was recorded as the MIC.

The MICs of all antimicrobial agents, but not of the detergents and dyes (see Tables 4 and 5), were also determined with the efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN; Sigma-Aldrich, St. Louis, MO) at a final concentration of 20 μg/ml.

AmpC overexpression. To demonstrate the involvement of AmpC expression in the β-lactam resistance of the EcDC64 isolate, β-lactam MICs were also determined in the presence of 100 μg/ml of cloxacillin. A plasmid construct named pAP-1 (Table 1), which consisted of the *ampD* gene from *E. coli* cloned into the pBGS18 plasmid, was also used. EcDC64 was electroporated with pAP-1, transformants were selected in 25 μg/ml of kanamycin, and the β-lactam MICs were determined and compared to those of EcDC64 carrying the empty vector.

PCR amplification and sequencing of *acrR*, *acrA*, *acrB*, and *tolC* efflux pump genes. Genomic DNA from EcDC64 was extracted from overnight cultures at 37°C by use of the MasterPure genomic DNA purification kit (EPICENTRE Biotechnologies, Madison, WI). The oligonucleotides used to clone efflux pump genes are listed in Table 2. Oligonucleotides were designed on the basis of the previously reported nucleotide sequence of the *E. aerogenes* AcrAB-TolC (21) (accession numbers AJ306389 and AJ306390) and the recently released *Enterobacter* sp. strain 638 complete genome (accession number CP000653). PCR was performed with the extracted genomic DNA in 50 μl of a mixture containing 50 ng of DNA template, 300 nM of each primer, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 5 μl of the polymerase buffer, and 2.6 U of Expand high-fidelity enzyme (Roche Diagnostics GmbH; Mannheim, Germany). After an initial denaturation step of 2 min at 94°C, amplification was performed over 25 cycles consisting of 15 s at 94°C, 30 s at the hybridization temperature (60°C, 55°C, 48°C, and 45°C for *acrA*, *acrB*, *tolC*, and *acrR* genes, respectively), and 1 min at 72°C for elongation, followed by a final extension step of 7 min at 72°C. The PCR product was purified by a HighPure PCR product purification kit (Roche Diagnostics GmbH; Mannheim, Germany) and then cloned into the pCR2.1-TOPO TA cloning vector according to the manufacturer's instructions (Invitrogen Corporation, CA). Sequencing was carried out with a *Taq* DyeDeoxi-Terminator cycle sequencing kit in an automatic DNA sequencer (377 ABI-Prism; Perkin-Elmer). Each product was sequenced on both strands.

Disposition of each of the gene of the operon (*acrR*, *acrA*, and *acrB*) was achieved by individual PCR products overlapping each other through the use of the indicated oligonucleotides (Table 2) (data not shown).

Nucleotide and amino acid sequence analysis was carried out by use of the following programs: ExPASy Proteomics Tools (http://www.ch.embnet.org/software/LALIGN_form.html) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of the *acrA* gene mutant. Disruption of the *acrA* gene in EcDC64 was performed by the method described by Datsenko and Wanner (6) with some modifications. The Red helper plasmid pKOBEG (gift from J. M. Ghigo) (4) (Table 1) is a low-copy-number plasmid that contains a gene for chloramphenicol resistance, a temperature-sensitive origin of replication, and the Red system, which includes three genes that express an exonuclease and the β and γ functions of phage λ. Plasmid pKOBEG (Table 1) was introduced into EcDC64 by heat shock, and transformants were selected on LB agar with chloramphenicol (20 μg/ml) after incubation for 24 h at 30°C. One transformant carrying the Red helper plasmid was made electrocompetent. A selectable kanamycin resistance gene was amplified by PCR from genomic DNA obtained from *E. coli* MC4100 (a gift from J. M. Ghigo) by use of primers that included 5′ extensions with homology for the *acrA* locus, i.e., acrAKm-F(forward) and acrAKm-R (reverse) in Table 2. The PCR product was used to disrupt the *acrA* gene of EcDC64(pKOBEG) by electroporation. Electroporation (25 μF, 200 Ω, 2.5 kV) of the EcDC64 electrocompetent strain was carried out according to the manufacturer's instructions (Bio-Rad Laboratories, Madrid, Spain) with 50 μl of cells and 1 μg of purified and dialyzed (0.025-μm nitrocellulose filters; Millipore, Billerica, MA) PCR product. Shocked cells were added to 1 ml of LB broth, incubated for 1 h at 30°C, spread onto LB agar with kanamycin (25 μg/ml), and then incubated at 30°C for 24 h. The EcΔ*acrA*::Km mutant strain was then grown on LB agar with kanamycin (25 μg/ml) at 43°C for 24 h and incubated overnight on LB agar with kanamycin (25 μg/ml) and LB agar with chloramphenicol (20 μg/ml) at 30°C to test for the loss of the helper plasmid.

To confirm the *acrA* deletion, a PCR assay with oligonucleotides (Table 2) that hybridize in the *acrA* gene was done. As expected, a band of 1,450 nucleotides (nt) was obtained by PCR, owing to the replacement of the *acrA* gene (1,194 nt) with the kanamycin resistance gene (1,450 nt).

Construction of pAP-2 and pAP-3 vectors. For pAP-2 construction (Table 1), two oligonucleotide primers (Table 2) were designed from the *acrA* sequence of *E. aerogenes* to amplify, by PCR, the *acrA* gene from EcDC64. The amplified DNA was purified, digested with EcoRV and HindIII, and then ligated (rapid DNA ligation kit; Roche Diagnostics GmbH, Mannheim, Germany) into a similarly digested expression pACYC184 vector under the control of the CTX-M-14 gene promoter. The CTX-M-14 gene promoter was first amplified with the pctx oligonucleotides (Table 2), designed from position 1502 to position 1740 of the sequence with EMBL database accession no. AF252622 from an *E. coli* clinical isolate harboring the $bla_{CTX\text{-}M\text{-}14}$ gene, and then cloned into the pACYC184 vector. The accuracy of the construct was checked by restriction analysis. The recombinant plasmid (pAP-2) was then introduced into the EcΔAcrA competent cells for complementation studies.

For pAP-3 construction (Table 1), the full *acrR* gene was amplified with

TABLE 2. Oligonucleotides used in the present study

Function	Primer	Gene	Procedure	Sequence (5′→3′)[b]
Cloning	acrA-F	*acrA*	Cloning	*aagatatc*ATGAACAAAAACAGAGGGTTAACG
	acrA-R	*acrA*	Cloning	*aaaagctt*TTAAGACTTGGTTTGTTCAGACTG
	acrB-F	*acrB*	Cloning	ATGCCTAATTTCTTTATCGATCGC
	acrB-R[a]	*acrB*	Cloning	RTGRTGRTCNACNGGRTGRTT
	tolC-F	*tolC*	Cloning	ATGCAAATGAAGAAA
	tolC-R	*tolC*	Cloning	TTAATGACGGAACGGATT
	acrR-F	*acrR* from *E. aerogenes*	Cloning	*aaggatcc*ATGGCACGAAAAACCAAACAA CAGG
	acrR-R	*acrR* from *E. aerogenes*	Cloning	*aagaattc*TCAGGCCATGACCTCAGAGC
	pctx-F	promoter CTX-M-14	Cloning	*aaaggatcc*CCATCAATAAAATTGAG
	pctx-R	promoter CTX-M-14	Cloning	*aagatatc*CTCAAACTCCCAATACG
	acrAKm-F	*acrA*	Knockout	GGCGTCGTGACGCTCAAATCCGAACCTCT CCAGATGACAACAGAACTACCGGGCCG CACCAAAGCCACGTTGTGTCTCAA
	acrAKm-R	*acrA*	Knockout	TGAAGCTACTTCCTGAGCCTTGACCTGCG CGCCAGGACGAACTTTTTGCAAACCAGT AATGCGCTGAGGTCTGCCTCGTG
Expression studies (RT-PCR)	rpoB-F[a]	*rpoB*	RT-PCR	CAGCCGCGAYCAGGTTGACTACA
	rpoB-R	*rpoB*	RT-PCR	GACGCACCGCAGGATACCACCTG
	ompC-F	*ompC*	RT-PCR	AGGTTAACGATCAACTGACCGG
	ompC-R	*ompC*	RT-PCR	AAATTTCAGACCGGCGAACGCC
	ompF-F	*ompF*	RT-PCR	AGTGGGAATATAACTTCCAGGG
	ompF-R	*ompF*	RT-PCR	TGCGTCACCGAATTTCAGGCC
	acrART-F	*acrA*	RT-PCR	GATTATGATTCTGCCTTGGCCG
	acrART-R	*acrA*	RT-PCR	CAATGCGACCGCTGATAGGGG
Operon assembly	aefA-F[c]	*aefA* from *E. cloacae*	Amplification of the *acrR-acrA* intergenic region	GTACATCGCGTTCTGCACGCG
	acrA2-R[c]	*acrA* from *E. cloacae*	Amplification of the *acrR-acrA* intergenic region	TGCCTTGACATCACCGCCTTC
	acrA3-F[d]	*acrA* from *E. cloacae*	Amplification of the *acrAB* intergenic region	CGACAAACAACAAGCGTCGGC
	acrB2-R[d]	*acrB* from *E. cloacae*	Amplification of the *acrAB* intergenic region	ATGATGATTATGGCGATCACCC

[a] Oligonucleotide degenerated where R is A or G; N is A, C, G, or T; and Y is C or T.
[b] Lowercase and italic nucleotides indicate restriction sites for cloning.
[c] Oligonucleotides used for amplification, cloning, and sequencing of the *acrR* gene and the *acrR-acrA* intergenic region of the operon from *E. cloacae* EcDC64. F, forward; R, reverse.
[d] Oligonucleotides used for amplification, cloning, and sequencing of the *acrAB* intergenic region of the operon from *E. cloacae* EcDC64. F, forward; R, reverse.

specific oligonucleotides acrR-F and acrR-R (Table 2). The amplified fragment of 650 bp was then digested with BamHI/EcoRI and ligated to the pBGS18 plasmid (26) under the control of the above-mentioned CTX-M-14 gene promoter. The accuracy of the construct was checked by restriction analysis. The recombinant plasmid (pAP-3) was then introduced into the EcDC64 competent cells for antimicrobial susceptibility studies.

Real-time RT-PCR. Real-time reverse transcription-PCR (RT-PCR) was carried out to determine the expression levels of the *ompC* and *ompF* porin genes as well as of the tripartite efflux component, *acrA*. In all cases, the expression levels were normalized to the *rpoB* housekeeping gene coding for the RNA polymerase beta subunit. Primers designed from sequences with accession numbers AJ316540, AJ316539, EF627524, and AJ854260 from the *ompF*, *ompC*, *acrA*, and *rpoB* genes, respectively, were used. Total RNA was isolated with TRIzol reagent (Invitrogen Corporation, CA) according to the manufacturer's instructions and treated with RNase-free DNase I (Invitrogen Corporation, CA). The concentration of RNA was determined spectrophotometrically. RNA (1 μg) was reverse transcribed into single-stranded cDNA by use of a Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The cDNAs were quantified by real-time PCR amplification with specific primers (Table 2) by use of a Light Cycler 480 SYBR green I master kit and a Light Cycler 480 instrument (both from Roche Diagnostics GmbH, Mannheim, Germany) with an initial incubation of 95°C for 10 min followed by 45 cycles of 10 s at 95°C, 20 s at 60°C, and 10 s at 72°C. The expression levels were standardized relative to the transcription levels of *rpoB* (a housekeeping gene) for each isolate.

Detection of the *acrA* gene in several *E. cloacae* isolates. To determine whether the AcrAB-TolC efflux pump is widespread among clinical isolates of *E. cloacae*, a PCR assay was carried out to detect the *acrA* gene in different *E. cloacae* isolates. Six genotypically different *E. cloacae* isolates (repetitive extragenic palindromic-PCR tested), which showed different antibiotic susceptibility patterns and which had been collected in the hospital during the previous 5 years, were used. The reactions were carried out with a 50-μl volume of a reaction mixture containing 1.5 mM of $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 300 nM of each primer, 50 ng of chromosomal DNA, 5 μl of the polymerase buffer, and 2.5 U of *Taq* polymerase. The primers of the *acrA*-coding region, acrA-F and acrA-R (Table 2), were used. Amplification reactions were submitted to the following program: initial denaturation (10 min at 94°C) followed by 30 cycles of denaturation (2 min at 94°C), annealing (1 min at 45°C), and extension (1 min at 72°C), with a single extension cycle of 10 min at 72°C.

Nucleotide sequence accession numbers. The nucleotide sequence data in the study will appear in the EMBL/GenBank/DDBJ nucleotide sequence database. The accession numbers are as follows: EF627524 for the *acrR*, *acrA*, and *acrB* genes and AM287288 for the *tolC* gene.

RESULTS AND DISCUSSION

Cloning, sequencing, and analysis of the AcrAB-TolC efflux pump genes of *E. cloacae* EcDC64. *Enterobacter cloacae* EcDC64 was a clinical isolate which showed an antibiotic multiresistance profile (see Table 4).

The *acrR*, *acrA*, *acrB*, and *tolC* genes from EcDC64 were amplified by high-fidelity PCR, cloned into the pCR2.1-TOPO

TA cloning vector, and sequenced. Sequence analysis showed that genes *acrR*, *acrA*, *acrB*, and *tolC* from *E. cloacae*, which were 654, 1,194, 3,147, and 1,485 bp long, encoded proteins of 218, 397, 1,048, and 494 amino acids, respectively. AcrR, AcrA, AcrB, and TolC proteins from EcDC64 displayed 79.8%, 84%, 88%, and 82% amino acid identities with the respective homologues of *E. aerogenes* (21) and showed similar levels of similarity with the homologues of other *Enterobacteriaceae*.

It should be noted that PCR experiments carried out with oligonucleotides designed on the *acrR* gene of *E. aerogenes* and hybridization studies with the full *acrR* gene of *E. aerogenes* as the probe failed to amplify the *acrR* gene. We therefore decided to amplify a region upstream of the putative location of the *acrR* gene on the genome map of *E. aerogenes*. For this purpose, we designed a forward oligonucleotide based on a consensus region of the *aefA* gene, which in turn was designed on the basis of that of *E. aerogenes* and the recently released genome of *Enterobacter* spp. (accession no. CP000653). Amplification experiments with the aefA-F and acrA2-R primers (Table 2) yielded an amplicon of ca. 1,600 bp containing the entire *acrR* gene from *E. cloacae* and the partial coding region of *aefA*. The *acrR* gene of *E. cloacae* showed 73.9% nucleotide identity with that of *E. aerogenes* (21), notably lower than that observed for the *acrA* and *acrB* genes, which might account for the negative results obtained with the initial approach.

Assembling the sequence of the entire locus revealed an organization similar to that described for *E. aerogenes* (21), with the 22-bp *acrA-acrB* intergenic sequence (amplified with primers acrA3 and acrB2 [Table 2]) identical to that of *E. aerogenes*. The *acrR* and *acrA* genes are also transcribed divergently, and in the upstream *acrA* region, a 141-bp *acrR-acrA* intergenic sequence showed the same *acrA* and *acrR* promoter boxes previously found in *E. aerogenes* (GenBank accession no. AJ306389). Indeed, by RT-PCR, high levels of *acrR* and *acrA* gene expression were detected for EcDC64 compared to what was found for *E. cloacae* JC 194 (data not shown).

Construction of the EcΔacrA strain. To investigate the role of the AcrAB-TolC efflux pump in drug resistance, an *acrA* knockout of EcDC64 was constructed (EcΔacrA) by replacing part of the *acrA* gene with a kanamycin resistance cassette. The knockout of *acrA* was confirmed by PCR mapping as described in Materials and Methods (data not shown). RT-PCR results confirmed that no *acrA* expression was detectable in EcΔacrA (Table 3). When the EcΔacrA strain was transformed with pAP-2, RT-PCR analysis showed that the *acrA* expression was restored by complementation (Table 3).

Involvement of the AcrAB-TolC efflux pump in MDR. Susceptibility testing was carried out with EcDC64 (with and without PAβN), EcΔacrA (with and without PAβN), EcΔacrA(pAP-2), EcΔacrA(pACYC184), EcDC64(pAP-3), and EcDC64(pBGS18) (Tables 4, 5, and 6).

The efflux pump inhibitor PAβN affected the MICs of and susceptibilities to several antibiotics tested, revealing the presence of active drug efflux in EcDC64.

Analysis of the EcΔacrA mutant revealed an antibiotic susceptibility profile similar to that of EcDC64 in the presence of PAβN, with some exceptions (Tables 4 and 5). The data suggest that other efflux systems in addition to AcrAB-TolC operate in *E. cloacae* EcDC64. In this regard, the clearest result was that obtained with fusidic acid (Table 5). It appears that the AcrAB-TolC efflux system in *E. cloacae* does not pump out fusidic acid, unlike what is seen for other species of *Enterobacteriaceae* (19, 27).

TABLE 3. RT-PCR analysis of *ompC*, *ompF*, and *acrA* gene expression

Gene	Strain	Relative expression[a]
ompF	*E. cloacae* JC 194	1
	EcDC64	0.2032
	EcΔacrA	0.1931
ompC	*E. cloacae* JC 194	1
	EcDC64	0.00235
	EcΔacrA	0.00252
acrA	*E. cloacae* JC 194	1
	EcDC64	490
	EcΔacrA	0.002
	EcΔacrA(pAP-2)	>1,000
	EcDC64(pAP-3)	300

[a] Relative expression is calculated as $2^{-\Delta CT}$, where $-\Delta CT$ is the ratio of the crossing point target value to the crossing point reference value. The target is the indicated bacterial isolate, whereas the reference is in all cases *E. cloacae* JC 194.

Concerning the various antibiotics, β-lactam MICs were almost unaffected in EcΔacrA, with some exceptions (Table 4). The MICs of erythromycin and clindamycin were dramatically reduced in the knockout *acrA* mutant, as were those of tetracycline, chloramphenicol, and linezolid, while there were modest decreases in the MICs of aminoglycosides, ciprofloxacin, tigecycline, and trimethoprim-sulfamethoxazole. Susceptibility to telithromycin, novobiocin, nalidixic acid, norfloxacin, and rifampin was also increased in EcΔacrA. Antibiotic resistance was almost fully restored when the AcrA polypeptide was overexpressed in EcΔacrA (Tables 4 and 5).

On the other hand, the antimicrobial susceptibility of EcΔacrA further increased (in some cases dramatically) in the presence of PAβN, which points to the role of additional efflux systems.

Analysis of the antibiotic susceptibility pattern and MICs of EcDC64(pAP-3) revealed low MICs and large inhibition zones with most antimicrobial agents and other compounds studied compared to those for EcDC64 (Tables4,5, and 6). However, the effect was lower than that obtained with EcΔacrA, a fact which may be associated with the higher levels of expression of the *acrA* gene in EcDC64(pAP-3) than in EcΔacrA (Table 3). These results could be explained regarding the role of AcrR as a specific secondary modulator to fine-tune the level of *acrAB* transcription (13).

Analysis of detergents and dyes revealed lower MICs of most of them for the *acrA* knockout mutant than for its isogenic parental strain. The MICs of detergents and dyes indicated that the AcrAB-TolC efflux system is efficient at removing these products, with the exception of Triton X-100 (Table 6).

***acrA* detection in several *E. cloacae* isolates.** The *acrA* gene was detected by PCR in six genetically unrelated *E. cloacae* isolates used in the study (data not shown). This strongly suggests that the AcrAB-TolC efflux pump is resident in the species.

Other antibiotic resistance mechanisms in *E. cloacae* EcDC64. To analyze the involvement of efflux pumps in MDR in EcDC64, the intrinsic background of antibiotic resistance mechanisms that may act synergistically with efflux pumps must also be taken into account. EcDC64 showed a broad spectrum

TABLE 4. Antibiotic susceptibility profiles (MICs) for the indicated bacterial isolates

Antibiotic[a]	MIC (μg/ml) for:							
	EcDC64	EcDC64 + PAβN[b]	EcΔacrA	EcΔacrA + PAβN[b]	EcΔacrA(pAP-2)	EcΔacrA(pACYC184)	EcDC64(pAP-3)	EcDC64(pBGS18)
AMP	>256	>256	>256	>256	>256	>256	>256	>256
PEN	>256	>256	>256	>256	>256	>256	>256	>256
AMC	>256	>256	>256	>256	>256	>256	>256	>256
PIP	>256	192	192	6	>256	>256	96	>256
CEF	>256	>256	>256	>256	>256	>256	>256	>256
FOX	>256	>256	>256	>256	>256	>256	>256	>256
CXM	>256	>256	>256	48	>256	>256	>256	>256
CAZ	>256	>256	>256	16	>256	>256	>256	>256
CTX	>256	>256	>256	8	>256	>256	>256	>256
IPM	>32	32	24	6	>32	>32	24	>32
MEM	>32	32	24	1	>32	>32	24	>32
ATM	>256	64	64	2	>256	128	64	>256
OXA	>256	48	96	4	>256	>256	>256	>256
ERY	>256	1	12	0.094	128	16	48	>256
CLI	>256	6	2	1	>256	4	32	>256
TET	>256	64	64	1.5	>256	64	192	>256
CHL	8	0.25	0.5	0.047	>256	>256	1	8
TOB	1.5	0.5	0.125	0.047	1.5	0.25	0.38	1
GEN	1.5	0.5	0.125	0.094	1	0.125	0.25	1
AMK	2	1.5	0.5	0.125	2	0.5	1	2
CIP	0.047	0.006	0.003	<0.002	0.094	0.004	0.003	0.032
TGC	0.75	0.125	0.125	0.064	0.75	0.5	0.19	0.38
LZD	>256	4	8	0.5	>256	4	>256	>256
SXT	0.125	0.094	0.012	0.032	0.75	0.016	0.064	0.094

[a] Abbreviations for antibiotics: AMP, ampicillin; PEN, penicillin; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; CEF, cephalothin; FOX, cefoxitin; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; OXA, oxacillin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; CHL, chloramphenicol; TOB, tobramycin; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; TGC, tigecycline; LZD, linezolid; SXT, trimethoprim-sulfamethoxazole.
[b] MICs were determined with PAβN at 20 μg/ml.

of antibiotic resistance, with high-level resistance to β-lactams (including imipenem and meropenem). It has previously been reported that the interplay between AmpC expression and the reduction of porin expression may have an important role in β-lactam (including carbapenem) resistance in *E. cloacae* (10). We therefore aimed to determine if this was also true for the EcDC64 clinical isolate.

MICs for EcDC64 and for EcDC64 transformed with the *ampD* gene from *E. coli* were determined as well as for EcDC64 in the presence of cloxacillin. In both cases, there was a dramatic reduction in some β-lactam MICs (amoxicillin-clavulanic acid, most of the cephalosporins, and carbapenems), and indeed the MICs of imipenem and meropenem decreased from >32 to 2 and from >32 to 0.75 μg/ml, respectively. We also aimed to determine whether or not a reduction of the expression of *ompC* and *ompF* genes occurred in EcDC64. Real-time RT-PCR enabled comparison of the levels of expression of *ompC* and *ompF* genes in the EcDC64 strain and in the carbapenem-susceptible *E. cloacae* JC 194. Overall, the results showed that the level of expression of *ompC* was extremely low and that the level of expression of *ompF* was also lower than that seen for strain JC 194 (Table 3). Altogether, the above results confirmed that AmpC overexpression and decreased porin expression were also present as resistance mechanisms in EcDC64.

Concluding remarks. The involvement of efflux mediated by the AcrAB-TolC system in the decreased susceptibility to several antibiotics of EcDC64 was demonstrated by different ap-

TABLE 5. Antibiotic susceptibility profiles, expressed as diameters of the inhibition zones (in mm) determined by the standard disk diffusion method, for the indicated bacterial isolates

Antibiotic[a]	Susceptibility profile (diameter of inhibition zone [in mm]) for:							
	EcDC64	EcDC64 + PAβN[b]	EcΔacrA	EcΔacrA + PAβN[b]	EcΔacrA(pAP-2)	EcΔacrA(pACYC184)	EcDC64(pAP-3)	EcDC64(pBGS18)
TEL	9	32	19	34	13	18	12	10
FUS	0	>40	0	26	0	0	0	0
NOV	0	20	16	22	0	14	0	0
NAL	24	27	33	40	12	30	29	21
RIF	0	26	13	28	0	8	10	10
NOR	26	40	40	42	24	38	35	27

[a] Abbreviations for antibiotics: TEL, telithromycin; FUS, fusidic acid; NOV, novobiocin; NAL, nalidixic acid; RIF, rifampin; NOR, norfloxacin.
[b] Concentration of PAβN, 20 μg/ml.

TABLE 6. MICs of detergents and dyes for bacterial isolates

Compound	MIC (μg/ml) for:					
	EcDC64	EcΔacrA	EcΔacrA(pAP-2)	EcΔacrA(pACYC184)	EcDC64(pAP-3)	EcDC64(pBGS18)
Sodium dodecyl sulfate	8,192	256	4,096	128	1,024	8,192
Sodium deoxycholate	8,192	256	4,096	256	512	8,192
Sodium cholate	>16,384	2,048	>16,384	2,048	4,096	>16,384
Triton X-100	>16,384	>16,384	>16,384	>16,384	>16,384	>16,384
Acriflavine	128	16	64	16	32	128
Crystal violet	4,096	512	1,024	256	1,024	4,096
Ethidium bromide	2,048	128	1,024	64	256	2,048

proaches. First, the MICs in the presence of the efflux pump inhibitor PAβN (20 μg/ml) significantly increased the susceptibility with respect to that of the wild-type strain. Second, knockout of the *acrA* gene increased susceptibility to many of the same compounds, although there were some differences which suggested the role of additional efflux systems. Third, transformation of EcDC64 with the *acrR* gene from *E. aerogenes* also reduced the MICs of most of the same antibiotics.

The role of the efflux pump in β-lactam resistance has not been clearly elucidated because the host strain used in the present study bears a class C β-lactamase in its chromosome, overexpression of which affects most β-lactams. However, there was a decrease in piperacillin, aztreonam, oxacillin, and carbapenem MICs when either the EcDC64 strain with PAβN or MICs in the EcΔacrA strain were analyzed.

The role of AcrAB-TolC-type efflux pumps in macrolide and quinolone resistance has previously been described for some species of *Enterobacteriaceae* as a major mechanism of resistance (1, 9, 11, 15, 16, 19, 21). A macrolide-specific ABC-type transporter was recently reported for *E. coli* (8). This efflux system may also operate in the clinical isolate under study, which would explain the difference between the MIC of erythromycin for EcDC64 with PAβN and that for EcΔacrA (from >256 to 1 and 12 μg/ml, respectively).

It is noteworthy that this mechanism may at least partly explain the "intrinsic" resistance of *Enterobacter* to some antibiotics used to treat gram-positive infections, such as oxacillin, linezolid, clindamycin, fusidic acid, novobiocin, and rifampin, although another efflux pump is probably involved in resistance to fusidic acid. In this regard, it has been demonstrated that the intracellular concentration of linezolid in strains of *E. coli*, *E. aerogenes*, and *Citrobacter freundii* is comparatively low due to the efficient efflux of the drug by the resistance-nodulation-division-type efflux pump (25).

Unlike the AcrAB-TolC efflux pump from *E. aerogenes* (5), which does not appear to pump out telithromycin, the clinical isolate EcDC64 increased the inhibition zone for this antibiotic (from 9 to 19 mm) when the *acrA* gene was deleted. As with *E. aerogenes*, a more dramatic effect was observed when MICs of EcDC64 were determined in the presence of an efflux pump inhibitor (PAβN) (Table 5).

As for the clinical isolate under study, the AcrAB efflux pumps in *Proteus mirabilis* and *Klebsiella pneumoniae* have been associated with decreased susceptibility to tigecycline (23, 28), although in the present case the increase in susceptibility in the *acrA* knockout isolate was lower (the knockout strain MIC was sixfold lower than that for the parental strain). It is interesting that Keeney et al. (7) have recently reported the involvement of AcrAB of *E. cloacae* in decreased susceptibility to tigecycline, although the values reported (16- to 32-fold reductions) are higher than those we have described here. These differences may be related to the level of AcrAB-TolC gene expression in the strains under study.

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