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Effect of Transcriptional Activators SoxS, RobA, and RamA on Expression of Multidrug Efflux Pump AcrAB-TolC in *Enterobacter cloacae*

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Control of membrane permeability is a key step in regulating the intracellular concentration of antibiotics. Efflux pumps confer innate resistance to a wide range of toxic compounds such as antibiotics, dyes, detergents, and disinfectants in members of the *Enterobacteriaceae*. The AcrAB-TolC efflux pump is involved in multidrug resistance in *Enterobacter cloacae*. However, the underlying mechanism that regulates the system in this microorganism remains unknown. In *Escherichia coli*, the transcription of *acrAB* is upregulated under global stress conditions by proteins such as MarA, SoxS, and Rob. In the present study, two clinical isolates of *E. cloacae*, EcDC64 (a multidrug-resistant strain overexpressing the AcrAB-TolC efflux pump) and Jc194 (a strain with a basal AcrAB-TolC expression level), were used to determine whether similar global stress responses operate in *E. cloacae* and also to establish the molecular mechanisms underlying this response. A decrease in susceptibility to erythromycin, tetracycline, telithromycin, ciprofloxacin, and chloramphenicol was observed in clinical isolate Jc194 and, to a lesser extent in EcDC64, in the presence of salicylate, decanoate, tetracycline, and paraquat. Increased expression of the *acrAB* promoter in the presence of the above-described conditions was observed by flow cytometry and reverse transcription-PCR, by using a reporter fusion protein (green fluorescent protein). The expression level of the AcrAB promoter decreased in *E. cloacae* EcDC64 derivates deficient in SoxS, RobA, and RamA. Accordingly, the expression level of the AcrAB promoter was higher in *E. cloacae* Jc194 strains overproducing SoxS, RobA, and RamA. Overall, the data showed that SoxS, RobA, and RamA regulators were associated with the upregulation of *acrAB*, thus conferring antimicrobial resistance as well as a stress response in *E. cloacae*. In summary, the regulatory proteins SoxS, RobA, and RamA were cloned and sequenced for the first time in this species. The involvement of these proteins in conferring antimicrobial resistance through upregulation of *acrAB* was demonstrated in *E. cloacae*.

Enterobacter cloacae is an important nosocomial pathogen responsible for various infections, including sepsis, infections of the respiratory tract and urinary tract, wound infections, and meningitis. Multiple antibiotic-resistant strains have caused outbreaks of infections in hospitals, usually in settings where seriously ill patients are housed, such as intensive care units (ICUs). These pathogens of concern in an ICU setting cause significant morbidity and mortality, and infection management is complicated by resistance to multiple antibiotics (44).

Efflux pumps confer innate resistance to a wide range of toxic compounds, such as antibiotics, dyes, detergents, and disinfectants in members of the family *Enterobacteriaceae* (35, 36). Therefore, efflux pumps participate, at least partly, in the ability of bacteria to adapt to diverse environments, in drug resistance mechanisms, and in bacterial pathogenesis.

Control of membrane permeability is a key step in regulating the intracellular concentration of antibiotics in *Enterobacteriaceae*. The expression of porins and efflux pump components is jointly controlled by several positive global regulators, which respectively decrease or enhance transcription of specific genes such as *acrAB* and *tolC*, directly or via a regulation cascade. The *acrAB* regulation mechanism must be examined to understand the physiological role of AcrAB. In *Escherichia coli*, the expression of *acrAB* may be subjected to multiple levels of regulation, and it is locally modulated by the AcrR repressor (23). At a global level, AcrAB is controlled by stressful conditions and by regulators such as MarA, SoxS, and Rob (37, 40). In addition to identifying the MarA/SoxS/Rob family, George et al. (13) also identified and characterized RamA, a member of the AraC/XylS family, for its role in conferring multidrug resistance (MDR) in *Klebsiella*. Most recently, the *ramA* gene was identified in *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Salmonella* Typhimurium, in which it may also be involved in MDR (9, 19, 52). These activators perform their function via binding to discrete but degenerate nucleotide sequences known as the *mar*-, *rob*-, or *soxbox* sequences located in the upstream region of regulated genes (including *acrAB*) (14, 18, 25, 26, 30). The expression of each regulator is controlled differently. In *E. coli*, the *marA* transcription is repressed by *marR* and derepressed through binding by compounds such as salicylate (45). The level of *soxS* is controlled by the activator *soxR*, in contrast to the repressor activity of *marR* on *marA*. Activation of *soxR* by superoxides and redox cycling compounds, such as paraquat, induces expression of soxS and the subsequent induction of the

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TABLE 1 Bacterial strains and plasmids used in the present study

Strain or plasmid	Features (resistance marker)	Source or reference
Strains		
E. cloacae		
EcDC64	MDR phenotype strain overexpressing the AcrAB-TolC efflux pump	33, 34
Jc194	Clinical isolate with basal efflux pump expression and a more susceptible resistance profile than strain EcDC64	33, 34
Ec*Δsox*S::Km	EcDC64 with the *soxS* gene disrupted with a kanamycin resistance marker	This study
Ec*ΔrobA*::Km	EcDC64 with the *robA* gene disrupted with a kanamycin resistance marker	This study
Ec*ΔramA*::Km	EcDC64 with the *ramA* gene disrupted with a kanamycin resistance marker	This study
E. coli		
BL21(DE3)	*E. coli* cell suitable for transformation and protein purification	Invitrogen
TG1	Susceptible *E. coli* strain used for cloning procedures	Invitrogen
Plasmids		
pACYC184	Cloning vector (chloramphenicol and tetracycline)	7, 39
pAcGFP1	Cloning vector (ampicillin)	Clontech
pUCP24	Cloning vector (gentamicin)	50
pKOBEG	Red helper plasmid (chloramphenicol)	8
pGFP	pACYC184 (chloramphenicol) containing the *acrAB* promoter fused to the gene coding for GFP	This study
pCR-BluntII-TOPO	Cloning vector (kanamycin)	Invitrogen
pTsoxS	pCR-BluntII-TOPO (kanamycin) containing the *soxS* gene under the control of its own promoter	This study
pTrobA	pCR-BluntII-TOPO (kanamycin) containing the *robA* gene under the control of its own promoter	This study
pTramA	pCR-BluntII-TOPO (kanamycin) containing the *ramA* gene under the control of its own promoter	This study
pUCsoxS	pUCP24 (gentamicin) containing the *soxS* gene under the control of its own promoter	This study
pUCrobA	pUCP24 (gentamicin) containing the *robA* gene under the control of its own promoter	This study
pUCramA	pUCP24 (gentamicin) containing the *ramA* gene under the control of its own promoter	This study
pGEM-t	Cloning vector (ampicillin)	Promega
pET-28	Expression vector (kanamycin)	Novagen
pETM-44	Modified pET-24d expression vector (kanamycin)	EMBL

soxRS regulon (11). Rob is an abundant protein that is expressed constitutively (3), and its activity is enhanced by decanoate (40). Regulation of *ramA* is provided locally by *ramR*, presumably through prevention of RamR binding to an operator sequence near *ramA* and the subsequent relaxation of RamR repression at the *ramA* promoter (1, 38). Furthermore, induction of *acrAB* by indole is regulated through RamA, independently of MarA, SoxS, and Rob (30). The main goal of the present study was to investigate the regulation of *acrAB*, as well as its activator-encoding genes *soxS*, *robA*, and *ramA* isolated from *E. cloacae*, in the presence of different stress agents such as salicylate, decanoate, tetracycline, and paraquat. In order to define this regulation system, the effect of the inactivation or artificial overexpression of *soxS*, *robA*, and *ramA* on the expression of *acrAB* and the multidrug resistance phenotype of *E. cloacae* was also investigated.

MATERIALS AND METHODS

Strains, culture media, and plasmids. The laboratory strains and plasmids used in the present study are listed in Table 1. The clinical isolates used were two clonally unrelated strains of *Enterobacter cloacae* isolated from two different patients: EcDC64 and Jc194 (33, 34). Both strains were isolated from a patient admitted to the A Coruña University Hospital (Spain). *Escherichia coli* strain TG1 was used for cloning procedures. All strains used in the study were maintained at −80°C in 15% (vol/vol) glycerol until use. The strains were grown on MacConkey agar plates (Becton Dickinson, Franklin Lakes, NJ), in Luria-Bertani (LB) broth, or on LB agar in the presence of 50 μg of ampicillin/ml, 25 μg of kanamycin/ml, 30 μg of chloramphenicol/ml, 4 μg of gentamicin/ml, or 30 μg of tetracycline/ml, when necessary. Salicylate (10 mM), paraquat (100 μM), or decanoate (10 mM) (all from Sigma-Aldrich, St. Louis, MO) were added to LB agar and broth media, as required.

PCR amplification, sequencing, and cloning of soxS, *robA*, and *ramA* efflux pump regulatory genes. Genomic DNA from EcDC64 was extracted from overnight cultures at 37°C by use of a genomic DNA purification kit (Promega Corp., Madison, WI). The oligonucleotides used to isolate, amplify, and clone the efflux pump regulatory genes are listed in Table 2. To isolate and amplify *soxS*, *robA*, and *ramA*, oligonucleotides were designed on the basis of the previously reported nucleotide sequence of *Enterobacter* sp. strain 638 complete genome (GenBank accession code CP000653). The upstream region of these genes was amplified by using two consecutive random and specific primers. The partially random primer arb.1F (5′-GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCCC, where N represents A, T, G, or C) was used in the first PCR to amplify anonymous fragments of DNA in the upstream region of the regulator genes. PCR conditions consisted of 94°C for 3 min, followed by 5 cycles of 94°C for 30 s, 30°C for 30 s, and 72°C for 1 min, followed by 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min, with a final 5-min extension step at 72°C. The DNA fragments generated were then used as a template for the second PCR in which the primer arb.2F (5′-GGCCACGCGTCGACTAGTAC) was used. The reaction was performed under the same conditions as the first PCR, except annealing was carried out at 55°C. The PCR product was purified from the gel, by use of a Geneclean kit (MP Biomedicals, Ohio), and was then sequenced. Sequencing was carried out with a *Taq* DyeDeoxi- terminator cycle sequencing kit in an automatic DNA sequencer (377 ABI Prism; Perkin-Elmer). Specific oligonucleotides (Table 2) were designed from the sequences obtained to amplify each regulator with its own promoter. Amplicons were then cloned into the pCR-Blunt II-TOPO cloning vector, according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA), to generate the recombinant plasmids named pTsoxS, pTrobA, and pTramA (Table 1).

Construction of knockout strains. Disruption of the *soxS*, *robA*, and *ramA* genes in strain EcDC64 was performed by the method described by Datsenko and Wanner (10), with some modifications. The Red helper pKOBEG (kindly donated by J. M. Ghigo, Institut Pasteur, Paris, France)

TABLE 2 Primers used in this study

Primer	Gene[a]	Procedure	Sequence (5′–3′)[b]
sox-F	*soxS**	Cloning	ATGTCNCATCAGCARATWATTCAG
sox-R	*soxS*	Cloning	TTAGTTGAGCTGGTGGCGGTA
soxUp	*soxS*	Cloning	CAGGGCCACCAGCGTGTGAATA
rob-F	*robA*	Cloning	ATGGATCAGGCTGGAATTATT
rob-R	*robA*	Cloning	TTAACGACGTACCGGAATCAG
robUp	*robA*	Cloning	GGTGACGTTTTTAACGTCCGGATCG
ram-F	*ramA*	Cloning	ATGATCAATCAGGAAGCTGA
ram-R	*ramA**	Cloning	TCAGTGSGYRCGRCTGTG
ramUp	*ramA*	Cloning	TTGTGCTGGCGAAACATACC
soxSKmF	*soxS*	Knockout	ACCATTTCGAATAGCCCGACTTCCTGGCGACCACATCAATGTTCAACGGCTGGTCGATAT GTTCATCAATCCATTCAATACATATGAATATCCTCCTTAG
soxSKmR	*soxS*	Knockout	AGTGCCCTTCCGGCAATACGCCGAACGCGTCGCCGATGGTGGCCAGAGGAATGCCGATAC GTTGCGCAATTTTAATGATC TGTGTAGGCTGGAGCTGCTTCG
robAKmF	*robA*	Knockout	GGCTATTCCAAGTGGCATCTGCAAAGGATGTTCAAGGATGTCACCGGTCATGCTATCGGT GCCTATATTCGCGCACGTCGTGTGTAGGCTGGAGCTGCTTCG
robAKmR	*robA*	Knockout	AACGCGCATCTGATGGCGGAACTCGGAGATCTGCTCCAGGGAGCAGGAGTAGCTCTGCGT GGTGCCGACCAGGTGCGTTT CATATGAATATCCTCCTTAG
ramAKmF	*ramA*	Knockout	CTACACCAGCCGTTACGCATCGAAGAAATTGCCCGCCACGCGGGTTACTCAAAATGGCAT TTACAGCGGCTGTTTATGCA TGTGTAGGCTGGAGCTGCTTCG
ramAKmR	*ramA*	Knockout	TGCGGGTAAACGTCTGCTGCGAGTCAAACCCGTAGCGCAGGCAGATGTCGTACACCCGCT CGTCTGACTCACGCAGATCG CATATGAATATCCTCCTTAG
pacrA-F	*acrAB* promoter	Cloning	TCAGCAACGAACTCACATTTATG
pacrA-R	*acrAB* promoter	Cloning	TAACCCTCTGTTTTTGTTCATATGT
pacrAgfp-F	p*acrA*::*gfp* construction	Cloning	CCAACGAACTCACATTTATG
pacrAgfp-F	p*acrA*::*gfp* construction	Cloning	AGTCGCGGCCGCTCACTTGTACAGC
soxSpET44F	*soxS*	Cloning	*acatgt*CGCATCAGCAAATTATTCAG
soxSpET44F	*soxS*	Cloning	*aagctt*TTAGTTGAGCTGGTGGCGGT
ramApET44F	*ramA*	Cloning	*ccatgg*TCAATCAGGAAGCTGAAGGG
ramApET44R	*ramA*	Cloning	*aagctt*TCAGTGCGCGCGGCTGTG
robApET28F	*robA*	Cloning	*catatg*GATCAGGCTGGAATTAT
robApET28F	*robA*	Cloning	*ctcgag*TTAACGACGTACCGGAATCA
acrA RT-F	*acrA*	RT-PCR	GCCTCTGGCGGTCGTTCTGAT
acrA RT-R	*acrA*	RT-PCR	AGAGGTTCGGATTTGAGCGTCAC
acrB RT-F	*acrB*	RT-PCR	GTGAGCGTCGAGAAATCGTCCA
acrB RT-R	*acrB*	RT-PCR	TACGGCTGATGGCGTCCTTCAT
rpoB RT-F	*rpoB**	RT-PCR	CAGCCGCGAYCAGGTTGACTACA
rpoB RT-R	*rpoB*	RT-PCR	GACGCACCGCAGGATACCACCTG
soxS RT-F	*soxS*	RT-PCR	GCGCAGGTTACTGCTGGCAGCG
soxS RT-R	*soxS*	RT-PCR	GCGGCGAAACACGCGGGAAA
robA RT-F	*robA*	RT-PCR	CGCGCGGCCCATTCTTGATA
robA RT-R	*robA*	RT-PCR	GAGTGCCGGCGTCAACGAGA
ramA RT-F	*ramA*	RT-PCR	CAAAGGCGAAAGTCTGGGGCG
ramA RT-R	*ramA*	RT-PCR	CCCGTAGCGCAGGCAGATATCG

[a] *, The oligonucleotide degenerated where R is A or G,Y is C or T, S is C or G, W is T or A, and N is A, T, C, or G.
[b] Nucleotides shown in lowercase italics indicaterestriction sites for cloning.

(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 comprises an exonuclease and the β and γ functions of phage λ. The pKOBEG plasmid (Table 1) was introduced in the strain EcDC64 by heat shock, and transformants were selected on LB agar with chloramphenicol, after incubation for 24 h at 30°C. One transformant carrying the Red helper plasmid was made electrocompetent. A selectable resistance gene was amplified by PCR from genomic DNA by use of primers including 5′ extensions with homology for the *soxS*, *robA*, and *ramA* genes listed in Table 2. The PCR product was used to disrupt the *soxS*, *robA*, and *ramA* genes of strain EcDC64 by electroporation. Electroporation (25 μF, 200 Ω, and 2.5 kV) of the electrocompetent strains was carried out according to the manufacturer's instructions (Bio-Rad Laboratories, Madrid, Spain), with 50 μl of a cell suspension and 1 μg of the purified and dialyzed PCR products. Dialysis was performed in order to remove the salts of purified PCR products using 0.025-μm-pore-size nitrocellulose membranes (Millipore, Billerica, MA). Shocked cells were added to 1 ml of LB broth, incubated overnight at 30°C, spread on LB agar containing kanamycin, and incubated for 24 h at 30°C. The mutant strains were then grown on LB agar containing kanamycin for 24 h at 44°C and incubated overnight at 30°C on LB agar containing kanamycin and chloramphenicol in order to test for the loss of the helper plasmid.

Construction of transcriptional reporter fusion. The oligonucleotides listed in Table 2 were designed to amplify a fragment of 526 bp that contained 506 bp upstream and 21 bp downstream of the ATG of the *acrAB* operon from *E. cloacae* EcDC64. The PCR product was double digested with BamHI and NcoI and fused to the gene encoding the green fluorescent protein (GFP; *acgfp1*) in the pACGFP1 vector (Table 1). The fragment containing the transcriptional fusion was amplified with specific oligonucleotides (Table 2), purified and digested with HindIII and BamHI, and then ligated with the T4 DNA ligase (Promega) into a similarly digested pACYC184 expression vector to generate the recombinant

TABLE 3 Antibiotic susceptibility profiles[a]

Strain and stimulus	Antibiotic susceptibility (inhibition zone diam [mm])				
	Erythromycin	Tetracycline	Telithromycin	Ciprofloxacin	Chloramphenicol
Jc194					
None	10	25	13	34	24
SAL	0	18	9	28	21
DEC	0	23	11	32	24
PQ	0	18	8	27	17
TET	0	11	8	27	11
EcDC64					
None	0	0	9	32	25
SAL	0	0	0	28	21
DEC	0	0	8	32	25
PQ	0	0	0	27	18
TET	0	0	0	32	24
Ec*ΔrobA*					
None	10	26	14	38	24
SAL	0	21	11	28	22
DEC	10	26	13	46	33
PQ	0	18	11	27	17
TET	0	14	10	26	16
Ec*ΔsoxS*					
None	9	23	14	34	24
SAL	0	18	11	29	25
DEC	8	22	12	32	25
PQ[b]	10	31	17	42	28
TET	0	16	11	29	15
Ec*ΔramA*					
None	10	25	15	37	25
SAL	0	19	12	28	23
DEC	9	22	12	31	27
PQ	0	18	12	32	18
TET	0	24	17	40	26

[a] Antibiotic susceptibility profiles, expressed as the diameters of the inhibition zones, were determined by the standard disk diffusion method for the bacterial isolates in the presence of various stimuli: 10 mM sodium salicylate (SAL), 10 mM sodium decanoate (DEC), 0.1 mM paraquat (PQ), or 4 μg of tetracycline/ml (TET). A value of "0" means that an inhibition zone around the disk (6 mm) was not detected.

[b] The concentration of paraquat used was 50 μM.

plasmid named pGFP (Table 1). Fusion was sequenced in order to verify the DNA sequence.

Cloning procedures for complementation assays. Recombinant plasmids were first constructed and then introduced in the knockout strains for complementation studies. For construction of these plasmids, universal primers M13 and M13Rv were used to amplify the *soxS*, *robA*, and *ramA* genes under the control of their own promoter regions from the pTsoxS, pTrobA, and pTramA recombinant vectors, respectively. The amplified DNA was purified, digested, and then ligated with the T4 DNA ligase (Promega) into the pUCP24 expression vector (Table 1), previously digested with the same enzymes. The PCR fragments containing *soxS* and *ramA* genes were cloned between BamHI and EcoRI sites, and the fragment containing the *robA* gene was cloned between the HindIII and PstI sites. The accuracy of the construct was checked by restriction analysis.

Antibiotic susceptibility testing. The susceptibility of various strains to the following antibiotics was determined by the standard disk diffusion method (Becton Dickinson) on Mueller-Hinton agar (Tables 3 and 4): telithromycin, ciprofloxacin, erythromycin, chloramphenicol, and tetracycline. The susceptibility to these antibiotics was also determined in the presence of salicylate (10 mM), decanoate (10 mM), paraquat (100 μM), or tetracycline (the MICs for the strains tested were determined, after a gradual, stepwise increase in the exposure to tetracycline) (Table 4).

Flow cytometry assays. To examine the effect of transcriptional regulators SoxS, RobA, and RamA on the multidrug efflux pump AcrAB-TolC in *E. cloacae*, expression experiments were performed with GFP as a reporter. Single colonies of each bacterial strain (harboring the pGFP plasmid) were inoculated into 10 ml of LB broth containing appropriate amounts of the selected antibiotics. After overnight incubation at 37°C and 180 rpm, the cultures were diluted 1:100 in LB medium. The cells were then incubated at 37°C with continuous shaking until they reached an optical density at 600 nm (OD_{600}) of 0.4 and were then diluted 1:100 in 2 ml of 0.9% saline solution. Induction experiments were performed to test the effects of sodium salicylate (SAL), sodium decanoate (DEC), paraquat (PQ), and tetracycline (TET) on *acrAB* expression. A 10 mM concentration of SAL, 10 mM DEC, 0.1 mM PQ, or the MIC of TET was added to the cultures. Fluorescence analysis was performed with a FACScan cytometer, and 50,000 cells were measured for each sample. The values obtained were calculated as fluorescence units relative to the control strain containing the reporter plasmid grown under the same conditions.

Real-time RT-PCR experiments. Real-time reverse transcription-PCR (RT-PCR) was carried out to determine the expression levels of the *soxS*, *ramA*, and *robA* regulator genes and the efflux components *acrA* and *acrB*. Specific primers designed from *soxS*, *ramA*, *robA*, *acrA*, and *acrB* sequences (GenBank accession code JQ727666 for *soxS* gene; JQ727667

TABLE 4 Antibiotic susceptibility profiles[a]

Antibiotic	Susceptibility profile (inhibition zone diam [mm]) for:														
	E. cloacae EcDC64										*E. cloacae* Jc194				
	EcDC64	Ec Δ*soxS*	Ec Δ*soxS*(pUCsoxS)	Ec Δ*soxS*(pUCP24)	Ec Δ*robA*	Ec Δ*robA*(pUCrobA)	Ec Δ*robA*(pUCP24)	Ec Δ*ramA*	Ec Δ*ramA*(pUCramA)	Ec Δ*ramA*(pUCP24)	Jc194	Jc194(pTsoxS)	Jc194(pTrobA)	Jc194(pTramA)	Jc194(pTΦ)[b]
ERY	0	9	0	9	10	0	10	10	0	11	10	0	0	0	8
TET	0	23	13	25	26	14	26	25	14	25	25	0	0	0	20
TEL	9	14	9	19	14	10	18	15	11	18	13	0	0	0	14
CIP	32	34	22	38	38	21	39	37	23	40	34	25	26	23	32
CHL	25	24	12	23	24	12	25	25	15	25	24	0	0	8	21

[a] Antibiotic susceptibility profiles, expressed as diameters of the inhibition zones, were determined by the standard disk diffusion method for the bacterial isolates indicated. Abbreviations: ERY, erythromycin; TET, tetracycline; TEL, telithromycin; CIP, ciprofloxacin; CHL, chloramphenicol. A value of "0" means that an inhibition zone around the disk (6 mm) was not detected.

[b] pCR-BluntII-TOPO (pTΦ) is the empty cloning vector.

for *robA* gene and JQ727668 for *ramA* gene) are listed in Table 2. Total RNA was isolated with a High-Pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. 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) according to the manufacturer's instructions. cDNA was quantified by real-time PCR amplification with specific primers (Table 2) by use of a LightCycler 480 SYBR green I master kit and a LightCycler 480 instrument (both from Roche Diagnostics) 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. In all cases, the expression levels were standardized relative to the transcription levels of *rpoB* (a housekeeping gene) for each isolate.

Cloning, overexpression, and protein purification. Full-length *soxS*, *robA*, and *ramA* genes were amplified by PCR from genomic DNA of EcDC64 strain with the primers listed in Table 2. A DNA fragment corresponding to the *robA* gene was cloned into the pET-28 expression vector (Novagen) between NdeI and XhoI restriction sites. The *soxS* and *ramA* genes were cloned to MBP (maltose-binding protein) into the pETM-44 expression vector (modified pET-24d, EMBL-made vector by Arie Geerlof) between NcoI and HindIII restriction sites. Fusion proteins with a His tag at the N-terminal region were expressed in conventional *E. coli* strain BL21(DE3) cells grown in LB medium supplemented with 40 μg of kanamycin/ml. Cells were grown at 37°C until the OD_{600} reached 0.6. Protein expression was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside; Calbiochem) to the culture, to a final concentration of 0.5 mM, followed by incubation for 5 to 6 h at 37°C. Cell cultures were harvested by centrifugation (6,000 × *g*, 20 min, 4°C). Cell pellets were resuspended in 10 ml of lysis buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) and sonicated in a Misonix S4000 sonicator. Bacterial lysates were pelleted at 30,000 rpm for 1 h at 4°C in a 60Ti rotor (Beckman). The supernatant was loaded onto a His-Trap HP 5-ml column (GE Healthcare-Amersham Biosciences) equilibrated in binding buffer (10 mM Tris-HCl [pH 8.0], 5 mM imidazole, 500 mM NaCl). The target proteins were eluted with 50 ml of elution buffer (10 mM Tris-HCl [pH 7.0], 150 mM imidazole, 500 mM NaCl).

DNA mobility shift assays. Electrophoretic mobility shift assays (EMSAs) were performed as previously described (2). The DNA promoter of the *acrA* gene was amplified by PCR from *E. cloacae* EcDC64 genomic DNA by use of suitable oligonucleotide primers (Table 2), and the purified PCR fragment was cloned into the pGEM-T vector (Promega). The presence of the desired promoter was confirmed by sequencing the DNA plasmid with the T7 and SP6 primers of the pGEM-T vector. A DNA probe was prepared by PCR amplification, with one of the primers labeled with digoxigenin (DIG) at its 5′ end and was then purified. DNA-protein reaction mixtures (20 μl) containing 25 ng of a DIG-labeled DNA probe and SoxS, RamA, or RobA proteins were incubated for 30 min at 37°C in EMSA buffer containing 20 mM Tris-HCl (pH 8), 50 mM KCl, 5% (vol/vol) glycerol, 1 μg of bulk carrier sperm salmon DNA, 0.5 mM 1,4-dithiothreitol, and 0.1 mg of bovine serum albumin per ml. DNA-protein complexes were visualized by separation on a 5% nondenaturing polyacrylamide gel (40 mM Tris-acetate [pH 8.0]) at 90 V for 3 h and were then transferred to a Biodine B nylon membrane (Pall Gelman Laboratory). DIG-labeled DNA-protein complexes were detected according to the manufacturer's protocol (Roche).

Nucleotide sequence accession codes. The GenBank accession codes for the nucleotide sequences determined in the present study are as follows: JQ727666 for the *soxS* gene, JQ727667 for the *robA* gene, and JQ727668 for the *ramA* gene.

RESULTS

Stressing compounds such as salicylate, decanoate, tetracycline, and paraquat modify the resistance phenotype of *E. cloacae*. To analyze the effect of salicylate, decanoate, tetracycline, and paraquat on the resistance profile of *E. cloacae*, two clinical isolates of *E. cloacae* were used: EcDC64 and Jc194. *E. cloacae* EcDC64 was isolated from a patient admitted to the ICU of the A Coruña University Hospital (northwestern Spain). The bacterial isolate displayed a MDR phenotype overexpressing the AcrAB-TolC efflux pump and lower permeability than strain Jc194 (with basal efflux pump expression level) (33, 34).

The resistance profile to several antibiotic families known to be good substrates for efflux pumps (such as macrolides, tetracyclines, or quinolones) was determined in both strains, by the standard disk diffusion method, in the presence or absence of the different compounds studied (Table 3).

The effect of these compounds on the resistance profile of *E. cloacae* was evaluated, and the results revealed that the susceptibility to a number of antibiotics belonging to different antibiotic families was reduced in both wild-type (WT) strains in response to the compounds. When the bacteria were incubated with salicylate and paraquat, a decrease in the susceptibility to all of the antibi-

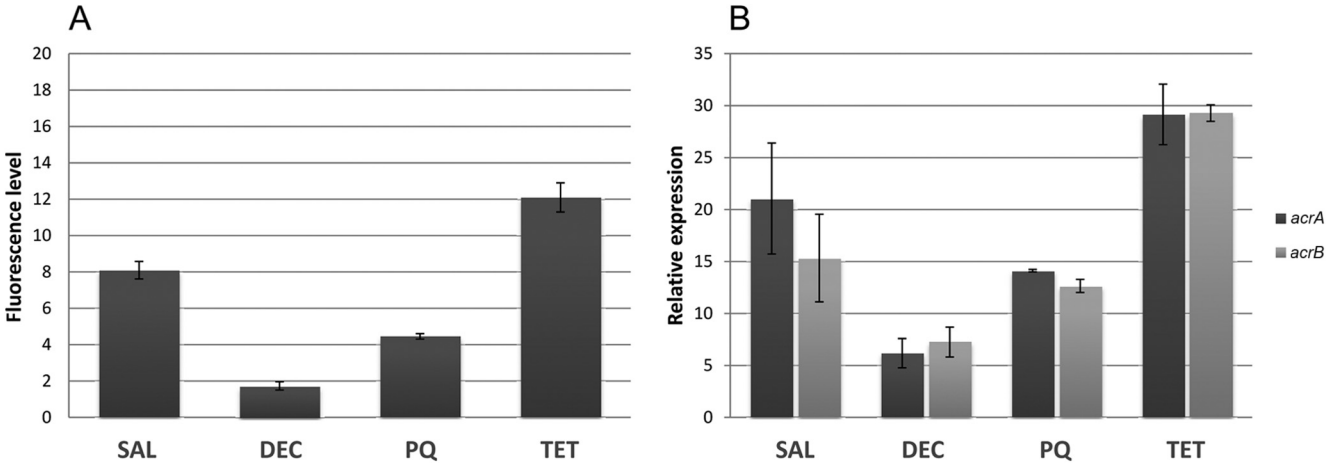

FIG 1 (A) Induction of the *acrAB* operon measured by growing *E. cloacae* Jc194 containing the reporter plasmid pGFP in the presence of 10 mM sodium salicylate (SAL), 10 mM sodium decanoate (DEC), 0.1 mM paraquat (PQ), or 4 μg of tetracycline/ml (TET). The values shown are relative fluorescence units, comparative to the control strain, Jgfp, grown without the compounds. The bars show the average values from triplicate assays. $P < 0.05$ in all cases. (B) RT-PCR analysis of *acrA* and *acrB* gene expression in *E. cloacae* Jc194 in the presence of 10 mM sodium salicylate (SAL), 10 mM sodium decanoate (DEC), 0.1 mM paraquat (PQ), or 4 μg of tetracycline/ml (TET). The bars show the average values for triplicate assays. The relative expression is calculated as $2^{-\Delta CT}$, where $^{-}\Delta CT$ is the ratio of the crossing points target value to the crossing point reference value. The target is the strain indicated, whereas the reference is *E. cloacae* Jc194 in all cases.

otics was observed in both WT strains (Table 3). The effect of tetracycline on antibiotic resistance of EcDC64 was not clearly established because of the high level of tetracycline resistance shown by this clinical isolate. However, this antibiotic was the strongest inducer of resistance in *E. cloacae* strain Jc194. The inhibition zones were reduced for all antibiotics, which indicate a significant increase in the resistance of this strain to the antibiotics.

Sodium decanoate had a modest effect on the antibiotic resistance of strain Jc194. The susceptibility to all antibiotics, except chloramphenicol, was slightly decreased. However, the resistance profile of EcDC64 was not modified by incubation of this strain with sodium decanoate. The intensity of the effect differed depending on the type of molecule (Table 3).

Analysis of *acrAB* expression in the presence of different stressing compounds. Flow cytometry assays were performed with the WT strain Jc194, which showed a basal expression level of the AcrAB-TolC efflux pump and a susceptible profile of resistance. Fluorescence data for *E. cloacae* Jc194 cultures containing the fusion *acrAB* promoter-GFP gene were measured in the presence of 10 mM sodium salicylate, 10 mM sodium decanoate, 4 μg of tetracycline/ml, and 0.1 mM paraquat. The results obtained are shown in Fig. 1A. All of the compounds tested induced an increase in the fluorescence intensity, indicating that the AcrAB efflux pump was upregulated in the presence of these compounds. The greatest increase in fluorescence was observed when *E. cloacae* strain Jc194 was incubated with tetracycline. A similar result was obtained in response to salicylate, although the increase in intensity was slightly lower. The compound that triggered the lowest expression of *acrAB* was sodium decanoate (Fig. 1A). To confirm the results of the *acrAB* expression levels obtained by flow cytometry experiments with GFP as a reporter, real-time RT-PCR assays were also performed. The results of the RT-PCR assays were found to be consistent with the flow cytometry data (Fig. 1B).

Role of SoxS, RobA, and RamA regulating AcrAB-TolC efflux pump in response to different signals. The *soxS*, *robA*, and *ramA* genes from EcDC64 were first amplified by high-fidelity PCR, cloned into the pCR-BluntII-TOPO cloning vector, and finally sequenced. Sequence analysis showed that genes *soxS*, *robA*, and *ramA* from *E. cloacae* EcDC64, which were 327, 870, and 375 bp long, respectively, encoded proteins containing 108, 289, and 124 amino acids, respectively. These proteins showed a high level of similarity to their homologues in other members of the *Enterobacteriaceae* family. When the sequences obtained were compared to the recently released genome of *E. cloacae* subsp. *cloacae* ATCC 13047 (GenBank accession code NC_014121), the amino acid identities were 100% for SoxS and 99% for RamA. RobA displayed 97% amino acid identity with the respective homologue in *E. cloacae* subsp. *cloacae* ATCC 13047. Moreover, the upstream region of these genes was amplified by using two consecutive random and specific primers, as described above. Fragments of various lengths (between 600 and 800 bp) were obtained. Analysis of the upstream region of *soxS* revealed the presence of the *soxR* gene. Assembly of the sequence of the entire locus revealed an organization similar to that described for other *Enterobacteriaceae*, with divergently transcribed *soxR* and *soxS* genes separated by an intergenic sequence of 98 bp. Amplification of the upstream region of *ramA* gene yielded an amplicon of ca. 700 bp containing the partial coding region of the *romA* gene.

In order to determine the different pathways activating the MDR *acrAB*-mediated phenotype, mRNA was extracted from *E. cloacae* Jc194 incubated in the presence of the compounds listed above, and the expression levels of the *soxS*, *robA*, and *ramA* genes were measured by real-time RT-PCR (Fig. 2). The results confirmed a high level of *soxS* expression under superoxide stress and showed that the sodium decanoate activates *robA* expression (Fig. 2). Furthermore, the *ramA* gene was overexpressed in *E. cloacae* Jc194 in the presence of tetracycline and salicylate (Fig. 2). Moreover, the expression level of *marA* gene was measured in the presence of salicylate and tetracycline, and the results obtained confirmed a high level of expression of *marA* gene by salycilate (data not shown).

Induction of *acrAB* by SoxS, RobA, and RamA in *E. cloacae* Jc194. To determine the effect of the regulatory proteins SoxS,

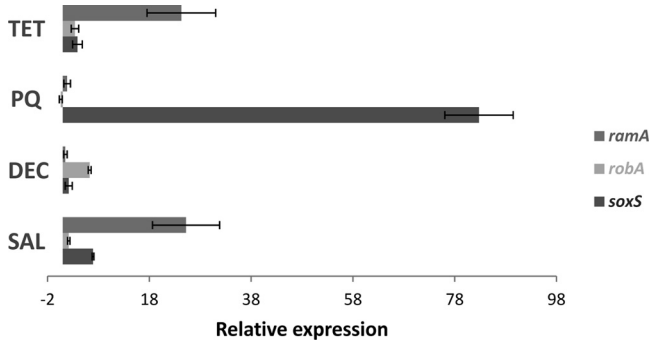

FIG 2 RT-PCR analysis of *soxS*, *robA*, and *ramA* gene expression in *E. cloacae* Jc194 in the presence of 10 mM sodium salicylate (SAL), 10 mM sodium decanoate (DEC), 0.1 mM paraquat (PQ), or 4 μg of tetracycline/ml (TET). The bars show the average values for triplicate assays. The relative expression was calculated as $2^{-\Delta CT}$, where $^{-}\Delta CT$ is the ratio of the crossing points target value to the crossing point reference value. The target is the strain indicated, whereas the reference is in all cases *E. cloacae* Jc194.

RobA, and RamA on the expression level of *acrAB*, fluorescence data of *E. cloacae* Jc194 cultures containing the *acrAB* promoter fused to the gene encoding for GFP protein were measured and quantified by flow cytometry.

The WT strain Jc194 harboring the recombinant plasmid pGFP (Table 1) was used as an experimental control that showed basal fluorescence intensity (fluorescence value = 1). Each of the genes encoding regulatory proteins was cloned under the control of their own promoter in the pCR-BluntII-TOPO cloning vector and introduced into the control strain Jc194 harboring the recombinant plasmid pGFP (Table 1) in order to measure the fluorescence intensity emitted when each of the regulators was overexpressed.

The results obtained by flow cytometry experiments showed that the regulatory proteins SoxS, RobA, and RamA induced the *acrAB* promoter, thus leading to a significant increase in fluorescence intensity (Fig. 3A). To confirm the results, the mRNA levels of *acrA* and *acrB* genes were measured by RT-PCR. The results of the RT-PCR assays are consistent with the flow cytometry data (Fig. 3B).

Influence of SoxS, RobA, and RamA on *acrAB* expression in *E. cloacae* EcDC64. In order to understand the role of the regulatory proteins SoxS, RobA and RamA in the MDR phenotype mediated by the AcrAB-TolC efflux pump in EcDC64 strain, *soxS*, *robA*, and *ramA* genes were inactivated in the wild-type EcDC64 strain to generate mutant strains EcΔ*soxS*, EcΔ*robA*, and EcΔ*ramA*. These knockout derivates were transformed with the recombinant plasmid (pGFP) carrying the transcriptional fusion of *acrAB* promoter-GFP gene. The WT EcDC64 strain was also transformed with pGFP to control for fluorescence intensity. Flow cytometry assays were performed with the WT strain and the knockout derivates harboring the pGFP recombinant plasmid, and fluorescence data were measured and recorded to investigate the effect of each regulator on the expression level of *acrAB* from *E. cloacae* EcDC64. The results obtained are shown in Fig. 4A. Inactivation of the *robA* gene, which is constitutively expressed (3), led to a significant reduction in the fluorescence intensity. The fluorescence intensity emitted by EcΔ*robA* was reduced by half relative to that observed in the WT strain EcDC64. However, when *soxS* and *ramA* genes were inactivated, the fluorescence intensity remained almost unchanged in comparison with that in the WT strain.

Complementation assays were performed to confirm the role of these regulatory proteins on *acrAB* expression. The *soxS*, *robA*, and *ramA* genes were amplified under the control of their own promoter regions from the WT EcDC64 strain and cloned into the pUCP24 vector (conferring resistance to gentamicin) in order to transform strains EcΔ*soxS*, EcΔ*robA*, and EcΔ*ramA*, respectively. The fluorescence activity of these cultures containing the pGFP recombinant plasmid was measured and quantified by flow cytometry. As expected, the fluorescence intensity was increased to various degrees when the regulatory genes were expressed from a plasmid into the knockout derivates (Fig. 4A). Real-time RT-PCR

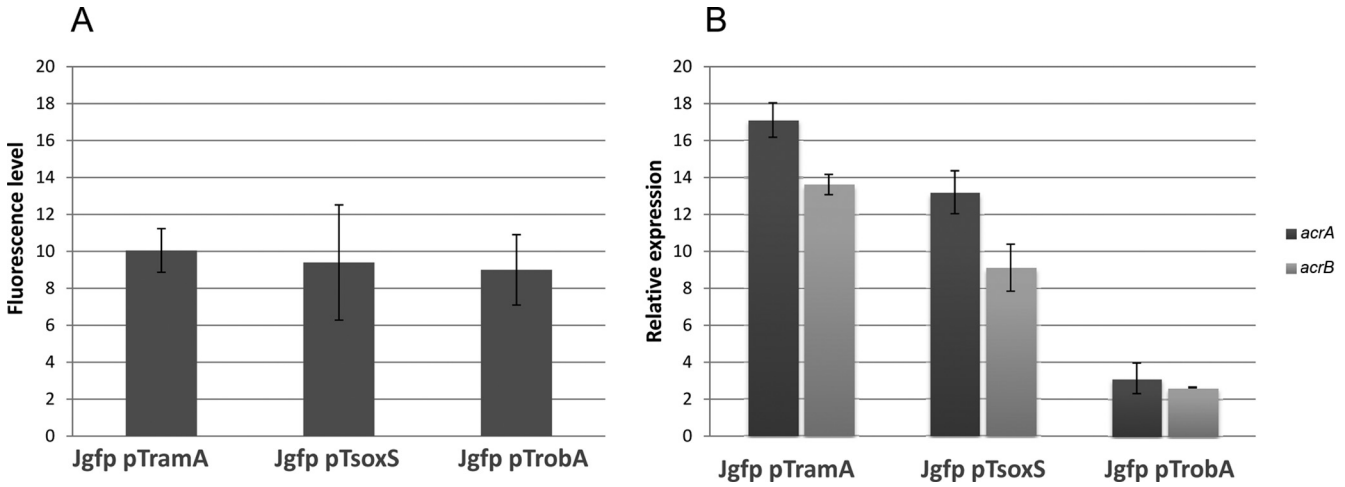

FIG 3 (A) Induction of *acrAB* operon due to *ramA*, *soxS*, and *robA* overexpression in *E. cloacae* Jc194 containing the reporter plasmid pGFP (see Materials and Methods), Jgfp. The values shown are fluorescence units relative to the Jgfp control. The bars show the average values from triplicate assays. $P < 0.05$ in all cases. (B) RT-PCR analysis of *acrA* and *acrB* gene expression in *E. cloacae* Jc194 overexpressing *ramA*, *soxS*, and *robA*. The bars show the average values from triplicate assays. Relative expression is calculated as $2^{-\Delta CT}$, where $^{-}\Delta CT$ is the ratio of the crossing points target value to the crossing point reference value. The target is the indicated strains, whereas the reference is *E. cloacae* Jc194 in all cases.

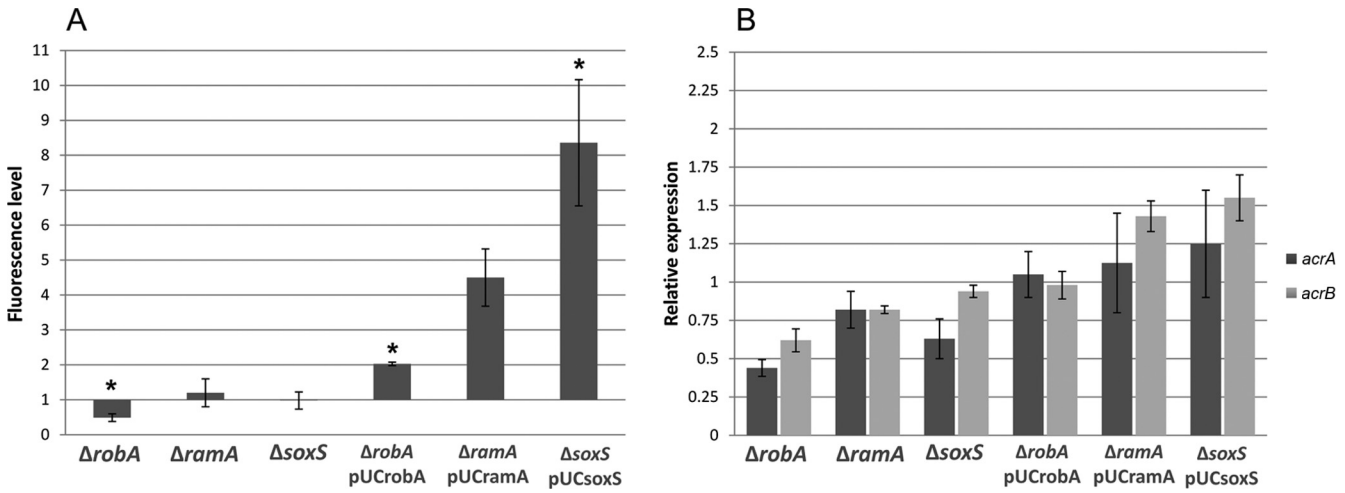

FIG 4 (A) Level of expression of *acrAB* in knockout strains from *E. cloacae* EcDC64 and their complemented strains. The values shown are fluorescence units relative to the EcDC64 WT strain containing the reporter plasmid, pGFP. The bars show the average values from triplicate assays. *, $P < 0.05$. (B) RT-PCR analysis of *acrA* and *acrB* gene expression in knockout strains from *E. cloacae* EcDC64 and their complemented strains. The bars show the average values from triplicate assays. The relative expression is calculated as $2^{-\Delta CT}$, where $^{-}\Delta CT$ is the ratio of the crossing points target value to the crossing point reference value. The target is the indicated strains, whereas the reference is *E. cloacae* EcDC64 in all cases.

results confirmed the fluorescence data obtained by flow cytometry assays (Fig. 4B).

Role of the *soxS*, *robA*, and *ramA* regulatory proteins on the antimicrobial resistance profile of *E. cloacae*. The resistance profile to several antibiotic families known as good substrates for efflux pumps (such as macrolides, tetracyclines, or quinolones) was determined in both strains by the standard disk diffusion method (Table 4). EcDC64 showed greater resistance or lower susceptibility to the antibiotics tested than strain Jc194, which is consistent with lower expression of AcrAB-TolC. Different approaches were used to determine the role of the regulatory proteins (SoxS, RobA, and RamA) on the efflux-mediated resistance profile.

First, the genes encoding regulatory proteins were overexpressed in *E. cloacae* isolate Jc194. The strains overexpressing *soxS*, *robA*, and *ramA* became resistant to erythromycin, tetracycline, and telithromycin and showed lower susceptibility to ciprofloxacin. The clinical isolate Jc194 was resistant to chloramphenicol when *soxS* and *robA* were overexpressed and showed reduced susceptibility to this antibiotic as a result of *ramA* overexpression. The strain harboring the empty cloning vector showed no significant changes in the resistance profile relative to the WT strain (Table 4).

On the other hand, the lack of the regulatory protein SoxS led to an increase in susceptibility to all antibiotics tested, except for chloramphenicol, where the inhibition zone determined by disk diffusion test remained unchanged relative to that observed for the WT strain. The MDR phenotype of isolate EcDC64 was restored or even increased for all antibiotics tested, except for tetracycline. The overexpression of *soxS* in Ec*ΔsoxS* led to partial restoration of the resistance to tetracycline by Ec*ΔsoxS*.

The same trend was observed in the isogenic Ec*ΔrobA* and Ec*ΔramA* isolates. The lack of RobA and RamA proteins had a similar effect on the resistance of EcDC64 isolate, with some exceptions (Table 4). For some antibiotics, such as tetracycline and ciprofloxacin, the increase in susceptibility was greater than that observed for Ec*ΔsoxS*. Analysis of knockout isogenic derivates Ec*ΔrobA* and Ec*ΔramA* overexpressing the *robA* and *ramA* genes, respectively, showed that the resistance profile was similar to that of the EcDC64 clinical isolate, although the MDR phenotype was not fully restored, as also observed in Ec*ΔsoxS*. Interestingly, the knockout derivates Ec*ΔsoxS*, Ec*ΔrobA* and Ec*ΔramA* containing the expression vector pUCP24 without any insertion were slightly more susceptible to the antibiotics than the other knockout derivates (Table 4). Moreover, susceptibility testing was performed with WT strains Jc194 and EcDC64 and the knockout derivates from EcDC64 (Table 3) in order to evaluate the effect of the lack of each regulator on the resistance profile in the presence of stressing agents.

In general, Ec*ΔsoxS* showed increased antibiotic resistance in response to the presence of salicylate, sodium decanoate and tetracycline. However, the superoxide stress generated by paraquat did not stimulate an increase in the resistance in *soxS* defective strain EcDC64. The antibiotic susceptibility also increased when Ec*ΔsoxS* was incubated with paraquat (Table 3). The concentration used in this case was 50 μM, which allowed the growth of Ec*ΔsoxS* and susceptibility testing to be performed. Analysis of antibiotic resistance profile of Ec*ΔrobA* mutant revealed that sodium decanoate, unlike salicylate, paraquat, and tetracycline, was not able to induce an increase in the antibiotic resistance of the *robA* defective EcDC64 clinical isolate.

Finally, tetracycline only induced an increase in the resistance to erythromycin by the isogenic knockout Ec*ΔramA*. Susceptibility to tetracycline was almost unchanged and increased susceptibility to telithromycin, ciprofloxacin and chloramphenicol was observed (Table 3). Although salicylate also induces overexpression of *ramA*, Ec*ΔramA* showed an increased antibiotic resistance in response to salicylate, due to the presence of a functional MarA regulator.

SoxS, RobA, and RamA bind to upstream region of *acrA* gene. The aforementioned results indicate that SoxS, RobA, and RamA activators play a major role in inducing *acrAB* expression in response to the presence of compounds such as salicylate, superoxides, decanoate, or antibiotics such as tetracycline. To under-

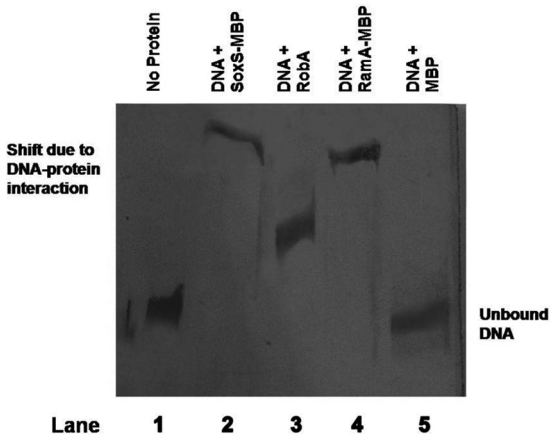

FIG 5 Electrophoretic mobility of the DNA fragments containing the EcDC64 upstream region of *acrA* gene from *E. cloacae* EcDC64. Lane 1, DIG-labeled DNA probe; lane 2, DIG-labeled DNA probe and SoxS protein fused to MBP; lane 3, DIG-labeled DNA probe and RobA protein; lane 4, DIG-labeled DNA probe and RamA protein fused to MBP; lane 5, DIG-labeled DNA probe and MBP protein.

stand the regulation of *acrAB* by these regulatory proteins, EMSAs with SoxS, RobA, and RamA were performed. Plasmids encoding the histidine-tagged proteins were constructed as described in Materials and Methods. SoxS and RamA proteins were purified after being fused to MBP (maltose-binding protein) to enhance their solubility. The MBP was also purified for use as a control. The upstream region of *acrA* gene was amplified by PCR, and the fragment was incubated with each of the proteins. The three proteins—SoxS, RobA, and RamA—from the *E. cloacae* EcDC64 isolate bound to the promoter of the *acrA* gene, as revealed by the shift in the gel migration, whereas MBP did not bind to the promoter (Fig. 5).

DISCUSSION

Multidrug efflux pumps are the major agents conferring drug resistance in bacteria. Several investigators have studied the important roles of the AcrAB-TolC efflux pump in bacterial drug resistance and virulence in *Enterobacteriaceae* (28, 35). The data currently available in *E. coli* and other *Enterobacteriaceae* show that multidrug efflux pumps are often expressed under precise and elaborate transcriptional control, including specific regulators such as AcrR, and global regulators such as MarA, SoxS, and Rob. In *E. coli*, the transcriptional activators belonging to the AraC/XylS family interact with AcrAB and effectively enhance efflux (15). Because the AcrAB-TolC plays a predominant role in the intrinsic resistance of *E. cloacae* to a wide range of antibiotics, dyes, detergents, and solvents, as well as in bacterial fitness and virulence (33, 34), study of its regulation is of great importance in understanding the action of antibiotics and bactericidal agents in *E. cloacae* and related organisms. The *mar*, *sox*, and *rob* regulons are well-characterized regulatory systems in *E. coli* (4, 21, 31, 51). It is thought that the influx and efflux in *E. cloacae* might be regulated in a similar way as in *E. coli*. Genomic analysis of *E. cloacae* EcDC64 revealed the presence of SoxS and RobA regulators, essentially identical to those in *E. coli*. The *ramA* regulon was found to show the same genetic organization as that observed in *Citrobacter* (41) and *Klebsiella* (5) species, in which the *ramA locus* is composed by *romA* and *ramA* genes, both controlled by the RamR repressor. The DNA-binding domains of SoxS, RobA, and RamA proteins from EcDC64 share a high level of sequence identity, which suggests that these proteins have overlapping specificity. These regulators activate the transcription of a large set of promoters, including the promoter of *acrAB*, through the direct binding to a degenerated and asymmetrical DNA sequence, known as the *marbox* sequence (24, 27). Analysis of the upstream region of the *acrAB* operon of *E. cloacae* (of both clinical isolates used in the present study) revealed a *marbox* sequence in a suitable position. The results presented here demonstrate the importance of SoxS, RobA, and RamA regulators in the modulation of AcrAB-TolC-mediated antibiotic resistance in *E. cloacae*.

Functional genomics approaches such as knockout studies or heterologous expression studies have been used to assess the effect of these transcriptional activators on *acrAB* expression. The *soxS*, *robA*, and *ramA* knockout strains derived from MDR *E. cloacae* EcDC64 were constructed. The *soxS*, *robA*, and *ramA* genes were also cloned into plasmids and overexpressed in the susceptible strain *E. cloacae* Jc194 under the control of their own promoter regions. The expression level of *acrAB* was measured by two different methods: flow cytometry using the *acrAB* promoter-GFP gene fusion as a reporter and real-time RT-PCR. In addition, susceptibility testing was performed with all of the different strains obtained (in which *soxS*, *robA*, and *ramA* were inactivated and overexpressed). Transcriptional activation of *acrAB* is the predominant cause of multidrug resistance in strains that overexpress MarA or the closely related global regulators SoxS, RobA, and RamA (6, 9, 12, 19, 20, 22, 32, 40, 43, 47, 51, 52). Chollet et al. (9) have described how an increase in *ramA*-mediated *acrAB* expression leads to an increase in resistance to tetracycline, chloramphenicol, quinolones, and β-lactams in *E. aerogenes*. In the same way, Hornsey et al. (17) described the emergence of AcrAB-mediated tigecycline resistance in *E. cloacae* through the RamA regulator. Our findings demonstrated that increased levels of *soxS*, *robA*, and *ramA* transcription activated *acrAB* expression, which led to an increase in antibiotic resistance in *E. cloacae* Jc194. The data indicated a correlation between the increased resistance to macrolides, tetracycline, ketolides, fluoroquinolones, and chloramphenicol and the expression levels of *acrAB* and *soxS*, *robA*, and *ramA*. The results of DNA mobility shift assays indicated that each of these three proteins directly control the expression of *acrAB* through binding to its upstream promoter region. Overall, the data indicated that SoxS, RobA, and RamA activators play a major role as activators of AcrAB-TolC expression. Overexpression of *ramA* caused the highest *acrAB* gene expression, followed by overexpression of SoxS and, to a lesser extent, RobA.

The differences in the *acrAB* expression levels were also illustrated by quinolone resistance in Jc194. The effects of RobA were generally weaker than those of RamA and SoxS for both antibiotic resistance and gene expression. In contrast to SoxS and RamA, RobA expression is constitutive (46), and it is inactive until activated by an induction signal (40, 42), which may explain the greater effect of the overexpressed *soxS* and *ramA* genes in the activation of the AcrAB-TolC efflux pump. On the other hand, the inactivation of the regulatory genes *soxS*, *robA*, and *ramA* in *E. cloacae* ECDC64 led to increased susceptibility to all antibiotics tested, except for chloramphenicol, in comparison to that of the WT strain, showing that the knockout acquires a similar antibiotic resistance profile to drug-susceptible strain Jc194. However, only inactivation of the *robA* gene had a slight effect on *acrAB* expres-

sion, which was reduced by half. These data, supported by the results obtained in the susceptibility analysis, suggest that RobA plays an important role in the antibiotic resistance of *E. cloacae* EcDC64. Although the inactivation of *soxS* and *ramA* did not affect the expression of *acrAB*, these proteins also affected the antibiotic resistance of isolate EcDC64. This may be due to the level of expression of *soxS* and *ramA* genes, which was significantly lower than that of the *robA* gene. Therefore, the effect of the inactivation of *soxS* and *ramA* on *acrAB* expression goes unnoticed when RobA is functional. In addition, these regulatory proteins control many other genes involved in antimicrobial resistance, which may explain the AcrAB-independent modification in the resistance profile of EcDC64 (27).

In *E. coli*, AcrAB-TolC is upregulated in response to different signals, such as aromatic weak acids (salicylate), superoxides (generated by paraquat), bile salts, fatty acids (decanoate), and tetracycline (49). These toxic compounds activate the transcription of global regulators, and they cause upregulation of the AcrAB-TolC efflux pump (11, 40, 45, 48). In the present study, we examined whether a similar mechanism occurs in *E. cloacae.* Indeed, the AcrAB-TolC system was upregulated by salicylate, decanoate, paraquat, and tetracycline, which affected the resistance profile of *E. cloacae*, as observed by a decreased susceptibility to a number of antibiotics. The results demonstrated that the induction profiles in *E. cloacae* are very similar to the induction profiles previously described in other species belonging to the *Enterobacteriaceae* family. The increased expression of *acrAB* was significantly correlated with the degree of resistance shown by *E. cloacae* Jc194 to different antibiotics, with some exceptions. Induction with tetracycline triggered the largest increase in resistance to all antibiotics tested. The lowest effect was observed after incubation of strain Jc194 with decanoate. The resistance to chloramphenicol was not modified in response to this compound. The increased levels of resistance to erythromycin, telithromycin, and ciprofloxacin, which were shown by Jc194 in response to salicylate, paraquat, and tetracycline, were similar, although the tetracycline had a greater effect on tetracycline and chloramphenicol resistance. Bacteria must distinguish among different stress conditions and respond in an appropriate manner. Each activator is regulated in response to a different signal, causing the final induction of *acrAB*. As described above, the AcrAB-TolC efflux pump in *E. cloacae* is upregulated by SoxS, RobA, and RamA, causing an efflux-mediated MDR phenotype in *E. cloacae.* The present data revealed that *acrAB* from *E. cloacae* is activated through different pathways depending on the stimulus applied. Salicylate induced *ramA* expression, in addition to activating *marA* transcription, as previously described in *E. coli* (45). *soxS* and *robA* expression was also moderately increased by salicylate in *E. cloacae*, unlike in *Salmonella*, in which *robA* is downregulated (16). The expression data supported by the susceptibility testing results suggest that salicylate induces activation of the AcrAB-TolC efflux pump, mainly through the MarA regulator. In *E. coli*, tetracycline induces *marA*, *soxS*, and *robA* expression, although the intensity of the effect differs (48). Tetracycline-mediated similar adaptation mechanisms of increased efflux in *E. cloacae* but, unlike *E. coli*, it activated *ramA* expression instead of *marA*. The effect of tetracycline on *ramA* expression was greater than the effect on *soxS* and *robA* expression, suggesting that the RamA activator plays a key role in the AcrAB regulation network. This hypothesis was supported by the antibiotic resistance profile shown by the *ramA*-defective *E. cloacae* EcDC64 isolate, in which antibiotic resistance was not induced by tetracycline. Furthermore, *E. cloacae* showed an oxidative stress response similar to that described in *E. coli*, in which *soxS* is highly overexpressed and *robA* is downregulated by paraquat (29), showing an increased resistance profile. However, paraquat increased the antibiotic susceptibility of *soxS*-defective *E. cloacae* EcDC64, which confirms the involvement of SoxS in the regulation of AcrAB under oxidative stress conditions. Rosenberg et al. (40) found that the induction of *acrAB* by decanoate requires Rob, but not MarA or SoxS, in *E. coli.* However, decanoate downregulated *robA* transcription and activated *marA* expression in *Salmonella enterica* serovar Typhimurium (16). We found a model for the *acrAB* regulatory network in *E. cloacae* similar to that in *E. coli*. Decanoate was not able to induce an increase in the antibiotic resistance of *robA*-defective EcDC64. Moreover, only RobA was upregulated by decanoate, which suggests that it mediates the regulation of AcrAB by free fatty acids such as decanoate, and it apparently helps the survival of *E. cloacae* by making it more resistant to antimicrobial agents.

In summary, the results presented here show that the AraC/XylS regulators SoxS, RobA, and RamA play an important role in efflux-mediated multidrug resistance in *E. cloacae* by increasing *acrAB* expression. It was further demonstrated that each activator is regulated in response to a different signal, causing the final induction of *acrAB*. Therefore, the AcrAB-TolC efflux pump in *E. cloacae* is activated through different pathways depending on the stimulus applied, thus conferring resistance to a variety of antimicrobial agents.

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