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Landscape of Resistance-Nodulation-Cell Division (RND)-Type Efflux Pumps in *Enterobacter cloacae* Complex

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In Gram-negative bacteria, the active efflux is an important mechanism of antimicrobial resistance, but little is known about the *Enterobacter cloacae* complex (ECC). It is mediated primarily by pumps belonging to the RND (resistance-nodulation-cell division) family, and only AcrB, part of the AcrAB-TolC tripartite system, was characterized in ECC. However, detailed genome sequence analysis of the strain *E. cloacae* subsp. *cloacae* ATCC 13047 revealed to us that 10 other genes putatively coded for RND-type transporters. We then characterized the role of all of these candidates by construction of corresponding deletion mutants, which were tested for their antimicrobial susceptibility to 36 compounds, their virulence in the invertebrate *Galleria mellonella* model of infection, and their ability to form biofilm. Only the Δ*acrB* mutant displayed significantly different phenotypes compared to that of the wild-type strain: 4- to 32-fold decrease of MICs of several antibiotics, antiseptics, and dyes, increased production of biofilm, and attenuated virulence in *G. mellonella*. In order to identify specific substrates of each pump, we individually expressed in *trans* all operons containing an RND pump-encoding gene into the Δ*acrB* hypersusceptible strain. We showed that three other RND-type efflux systems (ECL_00053-00055, ECL_01758-01759, and ECL_02124-02125) were able to partially restore the wild-type phenotype and to superadd to and even enlarge the broad range of antimicrobial resistance. This is the first global study assessing the role of all RND efflux pumps chromosomally encoded by the ECC, which confirms the major role of AcrB in both pathogenicity and resistance and the potential involvement of other RND-type members in acquired resistance.

The efflux systems are important mechanisms of drug resistance in bacteria. Five major groups of drug efflux transporter families have been identified so far: the RND (resistance-nodulation-cell division), MFS (major facilitator superfamily), MATE (multidrug and toxic compound extrusion), and ABC (ATP-binding cassette) families (1, 2). Those belonging to the RND family play a major role in resistance of Gram-negative bacteria to a wide range of toxic compounds, including antibiotics, biocides, and heavy metals (3, 4). These efflux systems consist of three components: the inner membrane protein (IMP), the periplasmic membrane fusion protein (MFP), and the outer membrane protein (OMP). The electrochemical potential of H^+ across cell membranes appears to be the driving force for drug efflux by RND family transporters (5, 6). The AcrAB-TolC tripartite efflux system is the most important one in Gram-negative bacteria, being involved in both intrinsic and acquired resistance to antibiotics, detergents, biocides, dyes, free fatty acids, and even solvents by constitutive expression and overexpression of the *acrAB-tolC* operon (1, 4, 7–9). In addition, *acrB* mutants in *Enterobacteriaceae*, selected *in vivo* and *in vitro*, are more susceptible to many antimicrobials (10, 11). The role of RND efflux pumps is not limited to the multidrug resistance (MDR) phenotype, since some of them have been shown to be required for virulence traits of different Gram-negative pathogens (11, 12). This explains why AcrB is considered the main clinically relevant RND system and constitutes a privileged target for the development of efflux pump inhibitor (EPI) molecules that could be used to treat infections (13–15). In *Enterobacteriaceae*, numerous other genes encoding RND pumps are present in the genomes, but their roles have been poorly investigated so far.

Species of the *Enterobacter cloacae* complex (ECC) are widely encountered in nature and also are part of the intestinal microbiota of both humans and animals (16). *E. cloacae* has taken on clinical importance during the last decade and has emerged as a redoubtable pathogen, accounting for up to 5% of hospital-acquired bacteremia, 5% of nosocomial pneumonia, 4% of nosocomial urinary tract infections, and 10% of postsurgical peritonitis cases (17, 18). ECC species are well adapted to the hospital environment and are able to develop MDR with very few therapeutic options. All ECC strains naturally possess the cephalosporinase-encoding *ampC* gene, the expression of which can be induced by some β-lactams, and it is responsible for the intrinsic resistance to ampicillin, amoxicillin-clavulanate, and first- and second-generation cephalosporins (19). The second chromosomal mechanism implicated in antimicrobial resistance in ECC is associated with the presence of the AcrAB-TolC efflux pump system (20).

Using the available genome sequence of *E. cloacae* subsp. *cloacae* ATCC 13047, we identified 10 genes coding for putative RND efflux pumps homologous to AcrB (21). The aim of this study was to characterize the 11 RND-type efflux systems by constructing the corresponding mutants and by performing *trans*-complementations using the *acrB* mutant strain. In addition to confirming the important role of AcrB in both antimicrobial resistance and viru-

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TABLE 1 Bacterial strains and plasmids used in the study

Strain or plasmid	Genotype/relevant characteristic(s)[a]	Reference or source
E. cloacae subsp. *cloacae* strains		
ECL13047	ATCC 13047	21
Δ*acrB*	ECL13047 derivative with deletion of *acrB* (ECL_01233)	This study
Δ00054	ECL13047 derivative with deletion of ECL_00054	This study
Δ01758	ECL13047 derivative with deletion of ECL_01758	This study
Δ01963	ECL13047 derivative with deletion of ECL_01963	This study
Δ02125	ECL13047 derivative with deletion of ECL_02125	This study
Δ02244	ECL13047 derivative with deletion of ECL_02244	This study
Δ03149	ECL13047 derivative with deletion of ECL_03149	This study
Δ03403	ECL13047 derivative with deletion of ECL_03403	This study
Δ03767	ECL13047 derivative with deletion of ECL_03767	This study
Δ04650	ECL13047 derivative with deletion of ECL_04650	This study
Δ04888	ECL13047 derivative with deletion of ECL_04888	This study
Δ*acrB*/Δ00054	Δ*acrB* derivative with deletion of ECL_00054	This study
Δ*acrB*/Δ01758	Δ*acrB* derivative with deletion of ECL_01758	This study
Δ*acrB*/Δ02125	Δ*acrB* derivative with deletion of ECL_02125	This study
Δ*acrB*/ECL_00053-55	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_00053-55	This study
Δ*acrB*/ECL_01233-1234	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_01233-1234	This study
Δ*acrB*/ECL_01758	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_01758	This study
Δ*acrB*/ECL_01960-63	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_01960-63	This study
Δ*acrB*/ECL_02124-25	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_02124-25	This study
Δ*acrB*/ECL_02243-44	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_02243-44	This study
Δ*acrB*/ECL_03149-50	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_03149-50	This study
Δ*acrB*/ECL_03401-04	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_03401-03	This study
Δ*acrB*/ECL_03767	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_03767	This study
Δ*acrB*/ECL_04649-50	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_04649-50	This study
Δ*acrB*/ECL_04888-93	ECLΔ*acrB* *trans*-complemented strain carrying pBAD202/D-TOPOΩECL_04888-93	This study
Plasmids		
pBAD202/D-TOPO	General expression vector with arabinose-inducible promoter, Kan^r	Life Technologies
pKOBEG	Recombination vector, phage λ recγβα operon under the control of the pBAD promoter, Cm^r	23
pKD4	Plasmid containing an FRT-flanked kanamycin cassette, Kan^r	24
pCP20_Gm	FLP-mediated recombination vector, Gen^r	25

[a] Cm^r, chloramphenicol resistant; Kan^r, kanamycin resistant; Gen^r, gentamicin resistant.

lence, we showed that other RND pumps also may have a role in MDR traits of this opportunistic pathogen.

(These results were presented in part at the 55th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA, 17 to 21 September 2015 [22].)

MATERIALS AND METHODS

Strains, media, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. The reference strain used was *E. cloacae* subsp. *cloacae* ATCC 13047 (ECL13047), the genome sequence of which is available already (GenBank accession numbers CP002886, FP929040, and AGSY00000000) (21). *Escherichia coli* and *Enterobacter cloacae* strains were cultured with shaking (200 rpm) at 37°C in LB medium.

Construction of the knockout mutants. The disruption of the genes encoding putative RND transporters was performed using the method previously described, with some modifications, using the Red helper plasmid pKOBEG (23, 24). This vector is a low-copy-number plasmid that contains a gene for chloramphenicol resistance selection, a temperature-sensitive origin of replication, and a gene encoding a recombinase. Briefly, pKOBEG first was introduced into the competent cells of ECL13047 by electroporation, and transformants were selected on LB agar with chloramphenicol (25 μg/ml) after incubation for 24 h at 30°C. A selectable kanamycin resistance cassette (flanked by flippase recognition target [FRT] sequences) was amplified by PCR using DNA of pKD4 plasmid as the template. The primers used included 5′ extensions with homology for the candidate genes (around 50 bases) (see Table S1 in the supplemental material). The PCR product was introduced into the ECL13047/pKOBEG plasmid by electroporation, and after homologous recombination, the disruption of the candidate gene was obtained. Selected clones were cured for the pKOBEG plasmid following a heat shock, creating the kanamycin-resistant variant. In order to have deletion mutants free of the antibiotic marker, strains then were transformed with the pCP20_Gm plasmid, which is able to express the FLP nuclease that recognizes the FRT sequences present on either side of the *kan* gene (25). Lastly, the mutants were verified by sequencing.

Construction of multicopy plasmid library containing putative RND transporter open reading frames (ORFs). The ECC RND efflux pump-encoding genes or operons and their promoters were amplified by PCR using primers listed in Table S1 in the supplemental material. Each amplicon was TA cloned into the overexpression plasmid, pBAD202 directional TOPO (Invitrogen, Courtaboeuf, France). *E. coli* TOP10 cells (Invitrogen) carrying pBAD202 recombinants containing correctly oriented inserts were selected on LB plates with 40 mg/liter kanamycin. After purification, each plasmid carrying an operon containing an RND efflux pump-coding gene was used to transform *acrB* mutant cells of ECL13047 (Δ*acrB* strain) (Table 1).

RNA manipulations. Total RNA was extracted from Δ*acrB*, Δ*acrB*/ECL_01233-34, Δ*acrB*/ECL_00053-55, Δ*acrB*/ECL_01758, and Δ*acrB*/ECL_02124-25 strains using the Direct-Zol RNA mini-prep kit (Zymo

TABLE 2 List of the RND transporters identified in the genome of *E. cloacae* ATCC 13047 based on homology with the sequence of AcrB

RND efflux gene	Gene name	Annotation	Size (bp/aa)[a]	% identity with *acrB* ECC	% positive for *acrB* ECC
ECL_00054	*eefB*	Multidrug efflux transport protein	3,111/1,036	58	75
ECL_01233	*acrB*	AcrB protein	3,147/1,048	100	100
ECL_01758		Acridine efflux pump	3,096/1,031	49	68
ECL_01963	*cusA*	Cu(I)/Ag(I) efflux system membrane protein	3,009/1,002	23	41
ECL_02125	*oqxB*	RND family multidrug efflux permease	3,153/1,050	41	62
ECL_02244		RND transporter, hydrophobe/amphiphile efflux-1 (HAE1) family	3,075/1,024	25	44
ECL_03149		Hydrophobe/amphiphile efflux-1 (HAE1) family protein	3,135/1,044	40	59
ECL_03403	*mdtC*	Multidrug efflux system subunit MdtC	3,078/1,025	29	50
ECL_03767		Aminoglycoside/multidrug efflux system	3,114/1,037	65	79
ECL_04650		Cation/multidrug efflux pump	3,114/1,037	80	89
ECL_04888	*cusA*	Cu(I)/Ag(I) efflux system membrane protein	3,147/1,048	24	43

[a] bp, base pair; aa, amino acid.

Research, Irvine, CA), and chromosomal DNA was removed by treating samples with the Turbo DNA-free kit (Life Technologies, Saint Aubin, France). RNA samples were quantified using the Biospec-Nano spectrophotometer (Shimadzu, Noisiel, France), and the integrities were assessed using an Agilent 2100 bioanalyzer. For reverse transcription-PCR (RT-PCR) experiments, cDNA was synthesized from total RNA (~1 μg) using the QuantiTect reverse transcription kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Transcript levels of the efflux pump-encoding genes were determined by the $\Delta\Delta C_T$ method (where C_T is threshold cycle) using the *rpoB* gene as a housekeeping control gene as previously described (19).

Drug susceptibility testing. MICs of different antibiotics, antiseptics, and biocides were determined by the microdilution method in Mueller-Hinton broth for all strains in three independent experiments (wild-type ECL13047, knockout deletion mutants, and *trans*-complemented ECLΔ*acrB* strains). The tested molecules were antibiotics (β-lactams [piperacillin, cefotaxime, cefoxitin, cefepime, imipenem, and ertapenem], aminoglycosides [gentamicin, tobramycin, and amikacin], fluoroquinolones [norfloxacin, levofloxacin, ciprofloxacin, and moxifloxacin], tetracyclines [tetracycline and tigecycline], chloramphenicol, co-trimoxazole, erythromycin, fusidic acid, nitrofurantoin, and colistin), antiseptics (benzalkonium chloride, cetylpyridinium bromide, chlorhexidine, tetraphenylphosphonium chloride), heavy metals (copper [$CuSO_4$], zinc [$ZnSO_4$], manganese [$MnSO_4$], mercury [$HgCl_2$], and silver [$AgNO_3$]), dyes (rhodamine 6G, acriflavine, acridine orange, crystal violet, ethidium bromide), sodium dodecyl-sulfate (SDS), and cathepsin E.

The MICs of levofloxacin, moxifloxacin, erythromycin, and chloramphenicol were determined for the wild-type, Δ*acrB*/ECL_00053-55, Δ*acrB*/ECL_01758, and Δ*acrB*/ECL_02124-25 strains with the efflux pump inhibitor (EPI) Phe-Arg-β-naphthylamide (PAβN; Sigma-Aldrich, St. Louis, MO) at final concentrations of 10, 25, and 50 μg/ml.

***In vitro* phenotypic assays.** For the H_2O_2 challenge, wild-type and mutant cells (log-phase cultures) were harvested at an optical density at 600 nm (OD_{600}) of 0.1 by centrifugation and resuspended in distilled water with 20 mM H_2O_2. These cultures were incubated at 37°C for 30 min. Before and after the challenge, samples were taken for plate counting. The number of CFU was determined after 24 h, 48 h, and 72 h of incubation at 37°C. Survival was determined as the ratio of the number of CFU after treatment to the number of CFU at the zero time point.

The capacity of each mutant to form biofilm was evaluated at 24 h, 48 h, and one week. Briefly, they were incubated in polystyrene 96-well microplates with LB medium and at 37°C. At the selected time point, the formed biofilm was colored with crystal violet (1%) and each well was scanned at 580 nm. Each value was the means from at least three experiments, and a statistical comparison of means was performed by using Student's test.

Virulence in *Galleria mellonella* model. The virulence of *E. cloacae* strains was tested in the *G. mellonella* model of infection. To this end, 10 μl of a suspension corresponding to an OD_{600} of 0.5 (i.e., $4.0 \times 10^8 \pm 1.0 \times 10^8$ CFU/ml) was injected dorsolaterally into the hemocoel of 20 larvae. After injection, the larvae were incubated at 37°C, and survival of the larvae was evaluated until 72 h postinfection. Each experiment was performed at least three times, and statistical comparisons were performed by using Student's test.

RESULTS AND DISCUSSION

Identification of RND-type drug transporter ORFs. AcrAB-TolC is a well-characterized multidrug efflux pump of the RND family in *E. cloacae* (20, 26, 27). Based on the sequence of the protein AcrB (designated ECL_01233 in ECL13047), the inner membrane transporter protein of the system, we searched for the presence of other members of this family of transporters. BLAST analysis allowed the identification of 10 additional loci on the chromosomal DNA of *E. cloacae* ECL13047 (Table 2 and Fig. 1). Sequence similarities to AcrB varied from 41% to 89%, while amino acid identities were between 23% and 80% (Table 2). To date, two transporters of a tripartite MDR efflux system have been identified (AcrB and EefB [ECL_00054]) in the genome of the ATCC reference strain (20, 28), whereas two others were annotated as belonging to the RND family (ECL_02125 and ECL_02244) (21). ClustalW alignments of all RND-type transporters revealed that three of the five residues corresponding to the amino acids essential for the proton transport within the AcrB identified in *E. coli* (D407, R970, and T977) were present in all sequences (29, 30). For the other two residues, K939 was lacking in ECL_02444, ECL_01963, and ECL_04888 strains, and D408 was not present in ECL_0196 and ECL_04888 sequences (data not shown). Note that these three sequences were those showing the weakest percent identity to the AcrB sequence (23 to 25%). Except for ECL_03767, the corresponding genes appeared to be included into operon structures whose promoter regions were determined *in silico* (Fig. 1). We also looked at the presence of the G288 residue in amino acid sequences of the different transporters. Indeed, it has been shown recently in *Salmonella* that a G288D substitution in AcrB altered its substrate specificity, conferring at the same time decreased susceptibility to CIP or TET and increased susceptibility to doxorubicin and minocycline (31). Among inner membrane RND antiporters identified in *E. cloacae*, only ECL_1758, ECL_3149, and ECL_3403 did not present such residues that may, at least in part, explain that these pumps could have different substrates (see below).

Phenotypic analysis of the RND drug transporter mutants. In

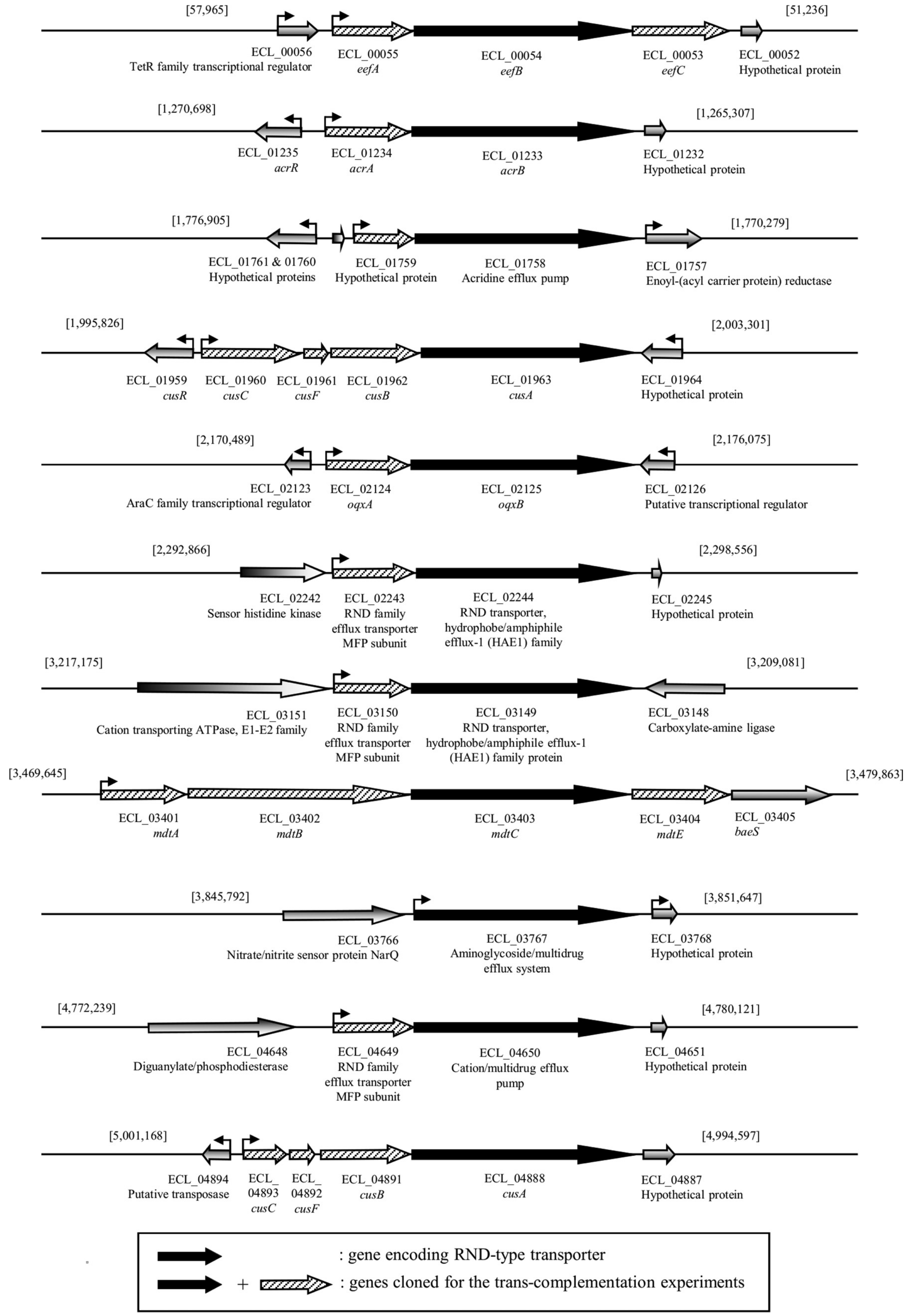


FIG 1 Genetic organization of the operons containing one gene coding for a putative RND efflux pump transporter homologous to AcrB.

order to characterize the role of the RND efflux pumps in *E. cloacae*, our first strategy was to construct individual deletion mutants of genes coding for the inner membrane proteins of the systems (that determine the substrate specificity of the efflux) and test them for their susceptibility to toxic compounds (antibiotics, antiseptics, biocides, heavy metals, and SDS), for their virulence, and for their ability to cope with different stresses. As expected, we showed that the *acrB* mutant was significantly more susceptible to several agents, such as antibiotics (such as fluoroquinolones, tetracyclines, co-trimoxazole, chloramphenicol, fusidic acid, and erythromycin), antiseptics (benzalkonium chloride and tetraphenylphosphonium chloride), dyes (rhodamine 6G, acriflavine, acridine orange, crystal violet, and ethidium bromide), and SDS (Table 3). It is worth noting that no differences in MICs were observed for β-lactams, aminoglycosides, and colistin. These results are in accordance with previous reports pointing out the importance of AcrB (and its overexpression) in antimicrobial resistance in *E. cloacae* (4, 27). Our data obtained with the other 10 mutants revealed that none of them showed an alteration in MICs of antimicrobial molecules tested (fold change of ≤4), likely because of the compensatory effect of AcrAB-TolC (data not shown). This confirmed that AcrAB-TolC is the unique RND-type efflux system involved in intrinsic resistance to toxic compounds (including antibiotics) in *E. cloacae*. Whatever the strain, we did not observe any modification in susceptibility to heavy metals, even for the two *cusA* mutant strains (ECL_01963 and ECL_04888), both encoding Cu(I)/Ag(I) efflux systems.

Another interesting feature of AcrAB is its role in the fitness and virulence of *E. cloacae*, as demonstrated for several other Gram-negative pathogens, such as *Salmonella* and *Klebsiella* (15, 26, 32, 33). Pérez and collaborators have shown that the inactivation of *acrA* or *tolC* significantly reduced the virulence of *E. cloacae* clinical isolates in an intraperitoneal mouse model of infection (26). As shown in Fig. 1, the *acrB* mutant was less virulent than the parental strain in the *G. mellonella* model. The *G. mellonella* immune response has strong similarities to the innate immune response of mammals, and this model of infection has been used extensively to evaluate the virulence of both Gram-positive and -negative pathogens (for a review, see references 34 and 35). Indeed, 100% of the larvae infected by the mutant were alive at 72 h postinfection, whereas 60% died when they were infected by the wild-type strain (Fig. 2). The involvement of AcrB in the pathogenicity of *E. cloacae* did not seemed to be linked to a role in the oxidative stress response or in the resistance to the antimicrobial peptide, since the corresponding mutant was not significantly more sensitive to H_2O_2 challenge (see Fig. S1 in the supplemental material) or to the presence of cathepsin E (MIC of 32 μg/ml and 16 μg/ml for the wild-type and Δ*acrB* strains, respectively), which are deleterious conditions encountered during the infectious process. The other RND pump mutants tested did not show virulence phenotypes altered from that of the parental counterpart (Fig. 2). However, we cannot exclude that some of them could be involved in the infection process that can be observed using other animal models of infection. In *Salmonella*, the ability to adhere and invade eukaryotic cells of a double mutation in genes encoding efflux pumps was lower than that in single *acr* mutants (13). Because of the double role of the RND-type pumps in antimicrobial resistance and virulence, it is obvious that such systems have to be privileged targets for the development of clinically usable inhibitors.

Surprisingly, the *acrB* deletion mutant (and not the others) seemed able to produce slightly but significantly more biofilm on polystyrene plates than the wild-type strain (Fig. 3). This result appeared contradictory to several studies showing that a defect in efflux activity (by mutation or using efflux inhibitors) impairs biofilm formation (for a review, see reference 15). It is well established that the expression of genes encoding efflux pumps is under tight regulation involving different transcriptional regulators, such as SoxS, RobA, and RamA, in *E. cloacae* (27). This argues for a general role of efflux pumps in various bacterial physiological processes. In this context, it can be suggested that the regulation leading to biofilm formation and linked to the efflux pumps (and inherent exchanges) in *E. cloacae* is different from that of *E. coli*, *Klebsiella*, or *Salmonella* (36–38).

***Trans*-complementation of the *acrB* mutant by other RND efflux pumps.** Studies using single-mutant strains clearly highlighted the major role of the AcrAB-TolC system among the numerous RND efflux pumps in intrinsic antimicrobial resistance. However, it is conceivable that the others also are functional, and in the mutant strain background, the loss of one pump may be complemented by one or more still present. This hypothesis was strongly supported by the fact that the antibiotic susceptibility profile of the Δ*acrA* mutant of *E. cloacae* EcDC64 was not identical to those observed for the wild type incubated in the presence of Phe-Arg-β-naphthylamide (PAβN), a very well-known EPI in Gram-negative bacteria (20). Moreover, it has been shown that the antimicrobial susceptibility of the Δ*acrA* mutant was further increased in the presence of PAβN (20). With the aim of evaluating the potential impact of the different systems in resistance to antimicrobial agents, we cloned individually all of the operons containing an RND pump into a multicopy plasmid. These plasmids were then used to assess whether each of the operons was able to complement the Δ*acrB* hypersusceptible strain.

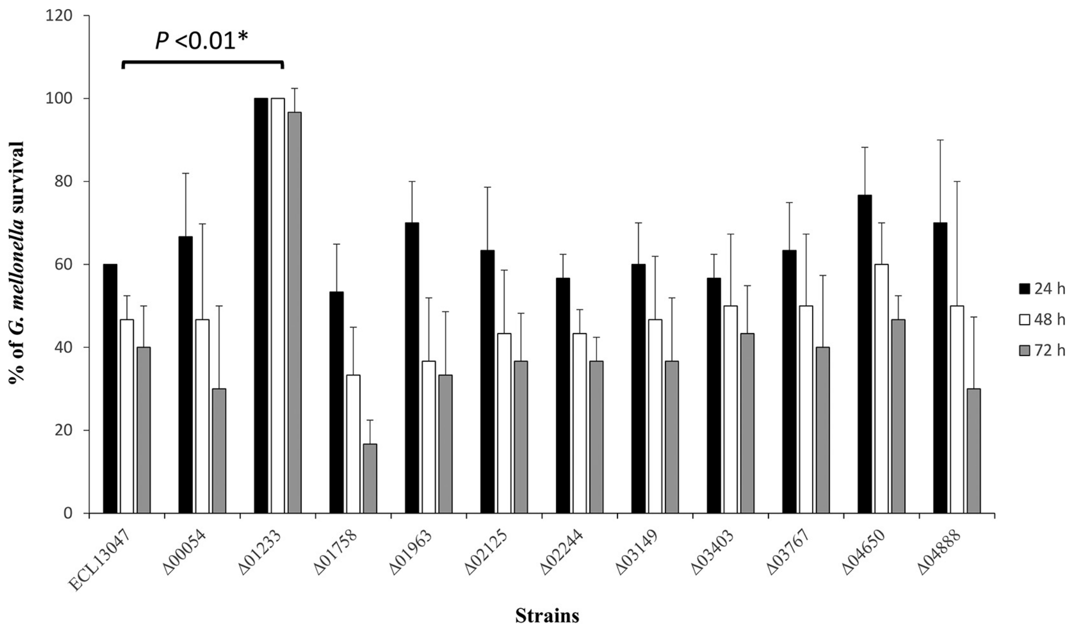
As expected, the introduction of the *acrAB* operon (where the RT-quantitative PCR [qPCR] results showed a C_T value of 13) into the Δ*acrB* mutant (where, of course, no transcription was detectable) completely restored the wild-type phenotype (Tables 3 and 4). On the other hand, *trans*-complementation with the operon ECL_01960-01963, ECL_02243-02244, or ECL_04888-04893 did not modify the antimicrobial susceptibility profile of the Δ*acrB* mutant (Tables 3 and 4). However, it is important to note that the three pumps of ECL_01963, ECL_02244, and ECL_04888 displayed the weakest similarity to AcrB (described above). Experiments carried out with the operons ECL_03401-03404 and ECL_03767 revealed a partial complementation of fusidic acid resistance. In addition, the expression of the ECL_03150-03149 and ECL_04649-04650 operons led to a weak restoration of MICs of benzalkonium chloride and erythromycin, respectively (Tables 3 and 4). It may be concluded that these efflux pumps have the aforementioned molecules as substrates but are not constitutively expressed and are not involved in intrinsic resistance. However, these systems could be inducible by some compounds (antibiotics or not) and even overexpressed in the case of the mutation/inactivation of their local/global regulators, as previously shown for MexCD-OprF and MexEF-OprN efflux systems in *Pseudomonas aeruginosa* (4).

Interestingly, three constructions (ECL_00053-00055, ECL_01758, and ECL_02124-02125) were able to largely restore the wild-type phenotype. We constructed the ECL_00054, ECL_01758, and ECL_2125 mutant strains in the *acrB*-deleted background. Except

TABLE 3 MICs of antibiotics, biocides, and metals for ECL13047 (wild type), Δ*acrB*, and Δ*acrB* transcomplemented strains carrying plasmids containing the different putative RND drug transporter ORFs

Compound[a]	MIC (μg/ml) for strain[b]:												
	ECL13047	Δ*acrB*	Δ*acrB*/ECL_01233-1234	Δ*acrB*/ECL_00053-55	Δ*acrB*/ECL_01758	Δ*acrB*/ECL_01960-63	Δ*acrB*/ECL_02124-25	Δ*acrB*/ECL_02243-44	Δ*acrB*/ECL_03149-50	Δ*acrB*/ECL_03401-04	Δ*acrB*/ECL_03767	Δ*acrB*/ECL_04649-50	Δ*acrB*/ECL_04888-93
Antibiotics													
Piperacillin	4	4	4	4	4	4	4	4	4	4	4	4	4
Cefotaxime	2	2	2	2	2	2	2	2	2	2	2	2	2
Cefoxitin	256	256	256	256	256	256	256	256	256	256	256	256	256
Cefepime	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032
Imipenem	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Ertapenem	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032
Gentamicin	0.125	0.25	0,125	0.25	**1 (4)**	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Tobramycin	0.5	0.25	0,125	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0,25	0.25
Amikacin	1	1	1	0.5	**4 (4)**	0.5	0.5	1	1	1	1	0.5	0.5
Norfloxacin	0.125	0.032	**0.125 (4)**	**0.125 (4)**	**0.125 (4)**	0.032	**0.5 (16)**	0.032	0.032	0.032	0.032	0.032	0.032
Levofloxacin	0.064	0.016	**0.064 (4)**	**0.25 (16)**	**0.125 (8)**	0.016	**0.25 (16)**	0.032	0.008	0.008	0.016	0.032	0.032
Ciprofloxacin	0.032	0.008	**0.032 (4)**	**0.064 (8)**	**0.064 (8)**	0.016	**0.125 (16)**	0.016	0.004	0.008	0.016	0.016	0.016
Moxifloxacin	0.064	0.008	**0.125 (16)**	**0.125 (16)**	**0.25 (32)**	0.008	**0.125 (16)**	0.008	0.008	0.008	0.008	0.016	0.008
Tetracycline	2	0.5	1	**2 (4)**	**4 (8)**	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Tigecycline	0.5	0.125	**0.5 (4)**	**1 (8)**	**2 (16)**	0.125	0.125	0.125	0.064	0.064	0.125	0.125	0.125
SXT	2	0.5	**2 (4)**	0.5	0.5	0.5	**32 (64)**	0.5	0.5	0.5	0.5	0.5	0.5
Chloramphenicol	8	0.5	**4 (8)**	**8 (16)**	**2 (4)**	1	**32 (64)**	1	1	1	1	1	1
Fusidic acid	256	4	**256 (64)**	**256 (64)**	**64 (16)**	4	**16 (4)**	4	8	**32 (8)**	**64 (16)**	4	4
Nitrofuratoin	32	32	32	32	32	32	64	32	64	32	32	64	32
Erythromycin	256	16	**256 (16)**	**256 (16)**	**256 (16)**	16	16	16	16	16	32	**64 (4)**	16
Colistin	256	128	128	128	128	128	128	128	128	128	128	128	128
Antiseptics													
Benzalkonium chloride	32	4	**32 (8)**	**16 (4)**	8	8	**16 (4)**	8	**16 (4)**	4	8	8	8
CTAB	16	16	16	16	16	8	16	16	16	16	16	16	16
Chlorhexidine	2	1	2	**4 (4)**	2	1	1	1	2	1	1	1	1
Tetraphenylphosphosnium	>1,024	128	**>1,024 (>8)**	**>1,024 (>8)**	**512 (4)**	128	**512 (4)**	128	256	64	64	128	128
Biocides													
Acridine	512	64	**512 (8)**	**256 (4)**	64	32	128	64	32	16	32	32	32
Acriflavine	64	8	**64 (8)**	**64 (8)**	16	8	**32 (4)**	8	4	4	8	8	8
Cristal violet	32	1	**16 (16)**	**16 (16)**	1	1	**4 (4)**	1	1	1	1	1	1
Ethidium bromide	1,024	64	**1,024 (16)**	**1,024 (16)**	128	64	**256 (4)**	32	128	256	32	32	64
Rhodamine	>1,024	128	**1,024 (8)**	**1,024 (8)**	128	128	**1,024 (8)**	128	128	128	128	128	128
Metals													
Copper	1,024	1,024	512	512	512	512	512	512	1,024	1,024	512	512	512
Zinc	128	128	128	128	128	128	128	128	64	128	128	128	128
Manganese	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	1,024	>1,024	>1,024	>1,024	>1,024
Silver	2	2	2	2	2	2	2	2	1	2	2	8	4
Mercury	4	8	8	8	8	8	8	8	8	8	8	8	8
Other													
SDS	1	0.004	**1 (256)**	**0.5 (128)**	**0.016 (4)**	0.004	**0.032 (8)**	0.004	0.004	0.004	0.004	0.004	0.004

[a] SXT, trimethoprim-sulfamethoxazole; CTAB, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate.
[b] Numbers in boldface are MICs that are significantly different (fold changes of ≥4 are indicated in parentheses) from the MICs of the Δ*acrB* mutant strain.

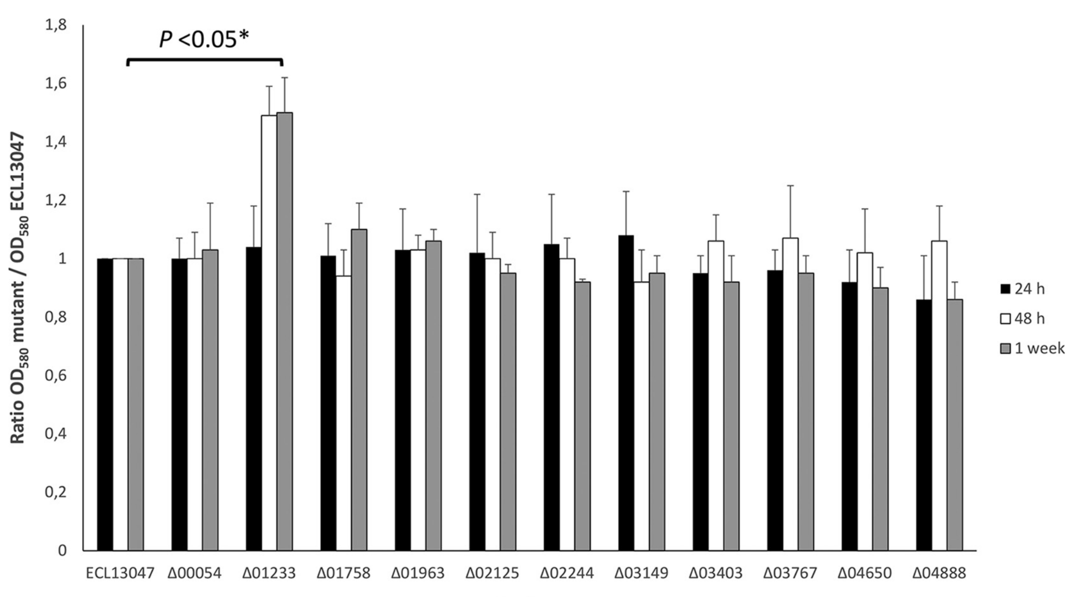


*P <0.001 at 24 h, P = 0.003 at 48 h, and P = 0.003 at 72 h

FIG 2 Effect of the deletions of efflux pump-encoding genes on virulence. Percent survival of *G. mellonella* larvae 24 h (black bars), 48 h (white bars), and 72 h (gray bars) after infection with around 4×10^6 CFU of *E. cloacae* bacterial cells per larva. Experiments were repeated at least three times, and the results represent the means ± standard deviations from live larvae.

for the Δ*acrB*/ΔECL_01758 and Δ*acrB*/ΔECL_02125 strains, which were 4-fold more sensitive to colistin and nitrofurantoin, respectively, than the Δ*acrB* mutant, the antibiotic resistance profiles of the three double mutants were similar to that of the Δ*acrB* single mutant (see Table S2 in the supplemental material). This again suggests that the absence of phenotype when these system were individually deleted very likely was due to the compensatory effect of the AcrAB-TolC activity.

The first construction, ECL_00053-00055, the transcription of which was 673-fold higher in the Δ*acrB*/ECL_00053-55 strain



*P = 0.01 at 48 h and P = 0.02 at 1 week

FIG 3 AcrB is involved in the biofilm formation of *E. cloacae*. The ability of *E. cloacae* ECL13047 and different mutant strains to form biofilm on polystyrene surfaces after 24 h (black bars), 48 h (white bars), and 1 week (gray bars) of incubation at 37°C is shown. OD values at 580 nm are from three independent experiments, and the means ± standard deviations are presented.

TABLE 4 Phenotypes of proven and putative efflux pump mutants of ECL13047[a]

RND efflux gene	Gene name	Deletion phenotype (agent[s], fold decrease in MIC)	Overexpression phenotype (agent[s], fold increase in MIC)
ECL_00054	*eefB*	No change	NOR (4), LEV (16), CIP (8), MOX (16), TET (4), TIG (8), CHL (16), FA (64), ERY (16), BC (4), CHH (4), TPP (>8), ACR (4), ACF (8), VC (16), EB (16), RHO (8), SDS (128)
ECL_01233	*acrB*	NOR (4), LEV (4), CIP (2), MOX (8), TET (8), TIG (4), SXT (4), CHL (16), FA (64), ERY (16), BC (4), CHH (2), ACR (16), ACF (8), VC (32), EB (16), RHO (>8), SDS (>64)	NOR (4), LEV (4), CIP (4), MOX (16), TIG (4), SXT (4), CHL (8), FA (64), ERY (16), BC (8), TPP (>8), ACR (8), ACF (8), VC (16), EB (16), RHO (8), SDS (256)
ECL_01758		No change	GMN (4), AKN (4), NOR (4), LEV (8), CIP (8), MOX (32), TET (8), TIG (16), CHL (4), FA (16), ERY (16), TPP (4), SDS (4)
ECL_01963	*cusA*	No change	No change
ECL_02125	*oqxB*	No change	NOR (16), LEV (16), CIP (16), MOX (16), SXT (64), CHL (64), FA (4), BC (4), TPP (4), ACF (4), VC (4), EB (4), RHO (8), SDS (8)
ECL_02244		No change	No change
ECL_03149		No change	BC (4)
ECL_03403	*mdtC*	No change	FA (8)
ECL_03767		No change	FA (16)
ECL_04650		No change	ERY (4)
ECL_04888	*cusA*	No change	No change

[a] Abbreviations: ACF, acriflavine; ACR, acridine orange; AKN, amikacin; BC, benzalkonium; CHH, chlorhexidine; CHL, chloramphenicol; CIP ciprofloxacin; CV, crystal violet; EB, ethidium bromide; ERY, erythromycin; FA, fusidic acid; GMN, gentamicin; LEV, levofloxacin; MOX, moxifloxacin; NOR, norfloxacin; RHO, rhodamine; SDS, sodium dodecyl sulfate; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TIG, tigecycline; TPP, tetraphenylphosphonium.

than in the Δ*acrB* mutant, corresponded to the homologous EefABC efflux pump identified in *Enterobacter aerogenes*, and the large panel of substrates of this RND system is detailed in Table 4 (39). It has been shown that the overexpression of *eefABC* in an *acrA* mutant of *E. aerogenes* also conferred the restoration of antibiotic MICs (39). In *E. aerogenes*, this operon is transcriptionally repressed by the H-NS (histone-like nucleoid-structuring) global regulator, and the activation of the *eefABC* promoter has been detected in chloramphenicol-resistant mutants (28, 39). In the *E. cloacae* genome, this operon was preceded by a gene coding for a TetR family transcriptional regulator (Fig. 1), the corresponding mutant of which seemed impaired in the colonization of *G. mellonella* and stimulated the expression of *eefABC* (unpublished results).

The second construction was ECL_01758, which complemented the hypersusceptibility phenotype of the *acrB* mutant, except in the cases of co-trimoxazole, benzalkonium chloride, and all biocides (Table 3). RT-qPCR results revealed that the transcription of ECL_01758 was 616-fold induced in the Δ*acrB*/ECL_01758 strain. Astonishingly, this transporter was annotated as an acridine efflux pump, whereas its expression did not increase resistance to this molecule (21). One of the most spectacular effects of the expression of ECL_01758 in the *acrB* mutant was the 4-fold increase of MICs of aminoglycosides (except tobramycin) that were not modified by the *acrB* deletion (Tables 3 and 4). These data showed that ECL_01758, when strongly expressed in the cell, can be part of the bacterial arsenal for developing resistance, especially to gentamicin and amikacin. Contrary to what we observed in the *acrB* mutant, Pérez et al. showed modest decreases in the MICs of aminoglycosides in the *acrA* mutant of *E. cloacae* (27). It has been reported that the overexpression of MexY correlated with decreased susceptibility to aminoglycosides of *P. aeruginosa* and that the RND protein AdeB of *Acinetobacter baumannii* was responsible for aminoglycoside resistance (40, 41). Interestingly, ECL_01758 was found to be the polypeptide most homologous to AdeB (58% identity and 76% similarity). Moreover, in the strain overexpressing ECL_01758, MICs of moxifloxacin and tigecycline were 4-fold higher than those of the wild-type strain (Table 3). Thus, it appeared that, in addition to the AcrAB pump, ECL_01758 and ECL_00054 had tigecycline as the substrate (Table 4). This is of clinical importance for the control of the emergence of tigecycline-resistant strains, since this molecule is increasingly used to fight against MDR *Enterobacter* (42–44)

Lastly, the plasmid carrying ECL_02124-02125 (annotated as *oqxAB* genes and transcriptionally induced 743-fold) restored the wild-type phenotype, except for tetracycline, tigecycline, erythromycin, and acridine orange (Tables 3 and 4). The MICs of the fluoroquinolones tested (except moxifloxacin) were even 4-fold higher than those of the wild-type strain (Tables 3 and 4). Similarly, MICs of co-trimoxazole and chloramphenicol were 16- and 4-fold higher in the *acrB* mutant harboring the ECL_02124-02125 operon than in the parental strain, respectively (Tables 3 and 4). It appears that this RND efflux system, when overexpressed by deregulation, can participate in reduced susceptibility to fluoroquinolones, co-trimoxazole, and chloramphenicol. In this context, it has been shown that the *oqxAB* chromosomal operon of *K. pneumonia* can become plasmid borne by transposition, leading to its overexpression and consequently exhibiting an MDR phenotype (45).

The susceptibility to levofloxacin, moxifloxacin, erythromycin, and chloramphenicol of ECL13047 significantly increased in the presence of PAβN (see Table S3 in the supplemental material). In addition, this EPI reduced the MICs to these antimicrobial molecules for the Δ*acrB*/ECL_00053-55, Δ*acrB*/ECL_01758, and Δ*acrB*/ECL_02124-25 *trans*-complemented strains (see Table S3), showing that the corresponding pumps also were targets of the inhibitor.

The present study confirmed the crucial role of the AcrAB-TolC efflux pump in both antimicrobial resistance and virulence of *E. cloacae* but also suggested that other operon-containing genes encoding RND efflux pump-mediated mechanisms complement, superadd to, and extend the range of antibiotic resistance. Besides AcrAB-TolC, it is probable that other members of the RND family are involved in the acquisition of additional resistance types (especially through overexpression), which may have an impact on opportunistic traits of *E. cloacae*. These results highlight the importance of pursuing the development of EPIs, especially those directed against the AcrB transporter.

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