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A *rob*-Like Gene of *Enterobacter cloacae* Affecting Porin Synthesis and Susceptibility to Multiple Antibiotics

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A chromosomal gene of *Enterobacter cloacae* affecting the synthesis of major outer membrane proteins in *E. cloacae* and *Escherichia coli* was cloned by using selection for resistance to cefoxitin in *E. coli*. The presence of the gene, when plasmid-borne, led to a decrease in the amount of porin F in *E. cloacae* and the amount of OmpF in *E. coli* and caused 2- to 32-fold increases in the MICs of chloramphenicol, tetracycline, quinolones, and β-lactam antibiotics. The gene encoded a 33-kDa protein, similar (83% identity) to the protein Rob involved in the initiation of DNA replication in *E. coli*, which was called RobA(EC1) by analogy. RobA from *E. cloacae* was found to inhibit *ompF* expression at the posttranscriptional level via activation of *micF*, a gene also apparently present in *E. cloacae*, as detected by PCR. As with its homolog from *E. coli*, RobA(EC1) is related to the XylS-AraC class of positive transcriptional regulators, along with MarA and SoxS, which also cause a *micF*-mediated decrease in the level of *ompF* expression.

The outer membranes of gram-negative bacteria contain porins which form water-filled channels allowing the diffusion of hydrophilic solutes (9, 17). The synthesis of these proteins is influenced by environmental stimuli which modulate the expression of specific sets of genes. In response to changes in osmolarity, the synthesis of the porins OmpF and OmpC of *Escherichia coli* is regulated at the transcriptional level by the products of the *ompB* operon, OmpR and EnvZ (16, 36). The *ompF* gene is preferentially expressed in media of low osmolarity, whereas *ompC* expression is increased in media of high osmolarity, with a simultaneous decrease in OmpF production. The translation of *ompF* mRNA is essentially controlled by *micF*, a regulatory gene located upstream of *ompC* and transcribed in the opposite direction. *micF* codes for a 4.5S RNA complementary to the 5′ terminal region of the *ompF* mRNA. *micF* RNA inhibits the translation of the *ompF* mRNA after binding to the ribosome-binding site and the translation start codon of the *ompF* transcript (1, 10, 30). The role of the *micF* gene is to regulate *ompF* expression under the control of the *ompB* operon (8, 30). Mutations at several loci, such as *tolC*, *marRAB* of the multiple antibiotic resistance operon, and *soxRS* of the redox-responsive regulon, cause induction of MarA and SoxS and result in the inhibition of *ompF* mRNA translation by *micF* transcripts (2, 5–7, 12, 29). *micF* may also be activated by overexpression of *rob* (3). One consequence of the overexpression of these genes in *E. coli* is a decrease in susceptibility to many β-lactam antibiotics (18) and various unrelated antibiotics (6, 20) and to heavy metal ions (31). However, the downregulation of *ompF* is not sufficient to fully explain the level of multiresistance observed (12, 32).

In *Enterobacter cloacae*, the overexpression of several genes, *romA* (23), *ompX* (39), and *ramA* (13), has been reported to result in multidrug resistance phenotypes associated with decreases in OmpF production, but details of the underlying mechanisms remain unclear. In an attempt to further understand the regulation of multidrug resistance in this species, we have cloned and partially characterized a gene which, when plasmid-borne, caused a decrease in the porin content of the outer membrane and in the susceptibility to several unrelated antibiotics.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains, bacteriophages, and plasmids used in the study are listed in Table 1. Luria broth or Mueller-Hinton (MH) agar (Sanofi Diagnostics Pasteur) was routinely used as the culture medium. For the preparation of outer membrane proteins and determination of the MICs, low-osmolarity medium A was prepared (23). All strains were cultured at 37°C with aeration.

Susceptibility testing and antibiotics. The MICs of antibiotics were determined, after serial twofold dilution in MH agar, by using a multiple-inoculum replicator and ca. 10^4 CFU per spot. The following antimicrobial agents were provided by their respective producers: chloramphenicol and cefotaxime, Hoechst Roussel Pharmaceuticals Ltd.; cefoxitin, imipenem, and norfloxacin, Merck Sharp & Dohme-Chibret; moxalactam, Eli Lilly & Co.; and nalidixic acid, Winthrop.

Gene cloning. Chromosomal DNA was extracted (4) from *E. cloacae* MD2 (25). After partial digestion with *Sau*3AI, fragments of ca. 2 to 10 kb were prepared by sucrose gradient centrifugation (4). The plasmid vector pHSS6 was digested with *Bam*HI and was dephosphorylated with calf intestine phosphatase (Boehringer Mannheim). Ligation was carried out at 16°C for 16 h with DNA ligase (Boehringer Mannheim). Recombinant DNA was transferred into *E. coli* AW737 by transformation (34), and resistant clones were selected on cefoxitin (8 μg/ml).

DNA hybridization, sequence analysis, and PCR conditions. DNA fragments were transferred, after agarose gel electrophoresis, to BiohylonZ$^+$ membranes (Bioprobe Systems, Montreuil, France). Prehybridization, hybridization, and washings were performed under high-stringency conditions by standard protocols (4). DNA probes were prepared from fragments excised from agarose gels, purified with the Gene Clean II Kit (Bio 101, Inc.), and radioactively labelled with [α-^{32}P]dCTP (3,000 Ci/mmol; Dupont) by using the Megaprime DNA labelling system (Amersham). The ca. 1-kb *Eco*RI-*Sma*I fragment of pAZ902 was ligated into M13mp18 and M13mp19 (41) (Boehringer Mannheim) and was sequenced by the dideoxy chain-termination method (35) with the T7 sequencing kit (Pharmacia P-L Biochemicals) and [α-^{32}P]dATP (3,000 Ci/mmol; ICN) and internal oligonucleotides. Nucleotide sequence analysis was facilitated by the use of the DNASIS program (Hitachi).

DNA amplification was carried out for 40 cycles in 100 μl of reaction mixture containing 10 ng of chromosome or plasmid DNA as template, 10 pmol each of the primers, 2 nmol (each) the deoxynucleoside triphosphates, and the reaction buffer for *Taq* DNA polymerase (Boehringer Mannheim). The DNA was denatured once for 3 min at 94°C, and each cycle was performed as follows: dena-

TABLE 1. Bacterial strains, bacteriophages, and plasmids used in the study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strain		
E. cloacae		
200	Clinical isolate, Cpm^s	25
MD2	Cpm^r mutant of strain 200 selected in vitro on moxalactam	25
E. coli		
AW737	*tonA31 tsx-78*	21
JM101	F′ *tra*Δ *36proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15*/ *supE thi* Δ(*lac-proAB*)	41
MH225	F$^-$ Δ(*argF-lac*)*U169 ara*Δ*I39 deoC1 fibB5301 ptsF25 relA1 rpsL150F*(*ompC-lacZ*$^+$)*10-25*	15
MH513	F$^-$ Δ(*argF-lac*)*U169 deoC1 fibB5301 ptsF25 relA1 rpsL150F*(*ompF-lacZ*$^+$) *16-13*	16
MH610	F$^-$ Δ(*argF-lac*)*U169 deoC1 fibB5301 ptsF25 relA1 rpsL150F*(*ompF-lacZ*$^+$) *16-10*	16
SM3001	MC4100 Δ*micF1*	27
JHC2205	MH610 Δ*micF1*	T. Nunoshiba
Plasmid		
pHSS6	Cloning vector, Km^r	37
pJSO4	Cmp^r *ompX*	39
pAZ901	pHSS6 containing a ca. 2-kb *Sau*3AI fragment from *E. cloacae* MD2 selected in *E. coli* AW737 on cefoxitin (8 μg/ml)	This study
pAZ902	Subcloned from pAZ901, containing a ca. 1 kb *Sau*3AI-*Eco*RI fragment from MD2, Cfx^r	This study

[a] Cmp^r, chloramphenicol resistant; Cpm^s, carbapenem susceptible; Cpm^r, carbapenem resistant; Cfx^r, cefoxitin resistant; Km^r, kanamycin resistant.

turation at 92°C for 1 min, annealing at 52°C for 2 min, and extension at 72°C for 2 min. The reaction was terminated by a final extension at 71°C for 10 min.

Outer membrane protein analysis. Outer membranes were prepared as described previously (14), and outer membrane proteins were separated on polyacrylamide gels (12%; wt/vol) containing sodium dodecyl sulfate (0.1%; wt/vol) and 3 or 5 M urea for better separation of the porins of *E. coli* and *E. cloacae*, respectively.

β-Galactosidase assay. The specific activity of β-galactosidase was assayed as described by Miller (28).

Nucleotide sequence accession number. The EMBL accession number for the sequence reported here is X87223.

RESULTS AND DISCUSSION

Cloning of an *E. cloacae* gene affecting the synthesis of outer membrane proteins in *E. cloacae* and *E. coli*. Chromosomal DNA cloned from *E. cloacae* MD2 into pHSS6 was transferred into *E. coli* AW737; transformants were selected on cefoxitin (8 μg/ml) and were screened for those not hydrolyzing nitrocefin in situ. DNA from one recombinant plasmid, pAZ901, was digested with *Eco*RI. Since one *Eco*RI restriction site was present in the cloned 2-kb fragment and the other was present in the polylinker of pHSS6 (37), the religated digest yielded pAZ902 with a ca. 1-kb insert (Table 1). The effect of the fragment cloned in pAZ902 on the synthesis of major outer membrane proteins was tested in strains *E. coli* AW737 and *E. cloacae* 200. After growth in low-osmolarity medium A, a clear decrease in the amount of OmpF, a slight increase in the amount of OmpC, but no change in the amount of OmpA was observed in *E. coli*, while a decrease in the amount of porin F and, to a lesser extent, in the amount of porin D was found in *E. cloacae* (Fig. 1). The presence of pAZ902 decreased the susceptibilities of both strains to several β-lactam and non-β-lactam antibiotics (Table 2). For *E. cloacae* 200 only, 4- and 32-fold increases in the MICs of imipenem and moxalactam, respectively, were observed, most likely resulting from the high-level cephalosporinase production in this strain (25) acting in synergy with the reduction in porin F-mediated outer membrane permeability for β-lactam antibiotics (24, 25, 33). Introduction of pAZ902 into *E. cloacae* 201 (24), which lacks porins F and D and for which the MICs of non-β-lactam antibiotics are similar to those for *E. cloacae* 200, resulted in two- to fourfold increases in the MICs of nalidixic acid, tetracycline, and chloramphenicol (data not shown). This suggests that a non-porin-mediated mechanism could also be involved in the multiple antibiotic resistance.

TABLE 2. MICs of various antibiotics for *E. coli* AW737 and *E. cloacae* 200 containing pAZ902

Strain	MIC (μg/ml)[a]							
	MOX	IMI	CXT	CTX	TET	CMP	NFLX	NAL
E. coli AW737	0.12	0.12	2	0.015	0.25	1	0.06	2
E. coli AW737 (pAZ902)	0.25	0.12	8	0.06	1	8	0.25	8
E. cloacae 200	4	0.25	>512	>512	2	16	0.125	4
E. cloacae 200 (pAZ902)	128	1	>512	>512	16	64	1	16

[a] MICs were determined on medium A agar containing the following antibiotics: moxalactam (MOX), imipenem (IMI), cefoxitin (CXT), cefotaxime (CTX), tetracycline (TET), chloramphenicol (CMP), norfloxacin (NFLX), and nalidixic acid (NAL).

Sequence analyses. To test whether the DNA fragment cloned in pAZ902 contained a gene related to *ompX* (39) or *romA* (23), Southern blot hybridizations were performed. With the 0.9-kb *Kpn*I fragment of pJSO4 used as a probe for *ompX* (39) and a probe for *romA* obtained after amplification of a chromosomal DNA fragment of *E. cloacae* MD2 with synthetic oligonucleotides (5′-AGGAATTCTCTTCATCTGCGTGGT-3′ and 5′-GTATCGATCACCCGAACGGGCTCG-3′ [23]), no hybridization was observed. The whole, 1,031-bp *Sau*3AI-*Eco*RI fragment of pAZ902 was sequenced (Fig. 2). It contained an open reading frame with a coding capacity for a protein of 289 amino acids and a deduced M_r of 33,085. This protein was screened for amino acid homology with translated

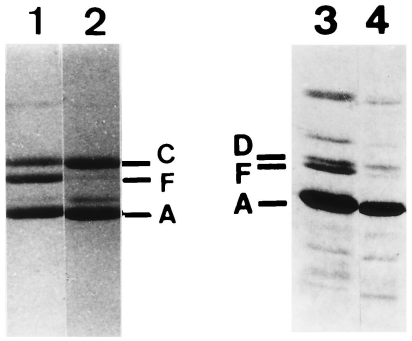

FIG. 1. Urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins prepared from *E. coli* AW737 (lane 1), *E. coli* AW737 (pAZ902) (lane 2), *E. cloacae* 200 (lane 3), and *E. cloacae* 200(pAZ902) (lane 4).

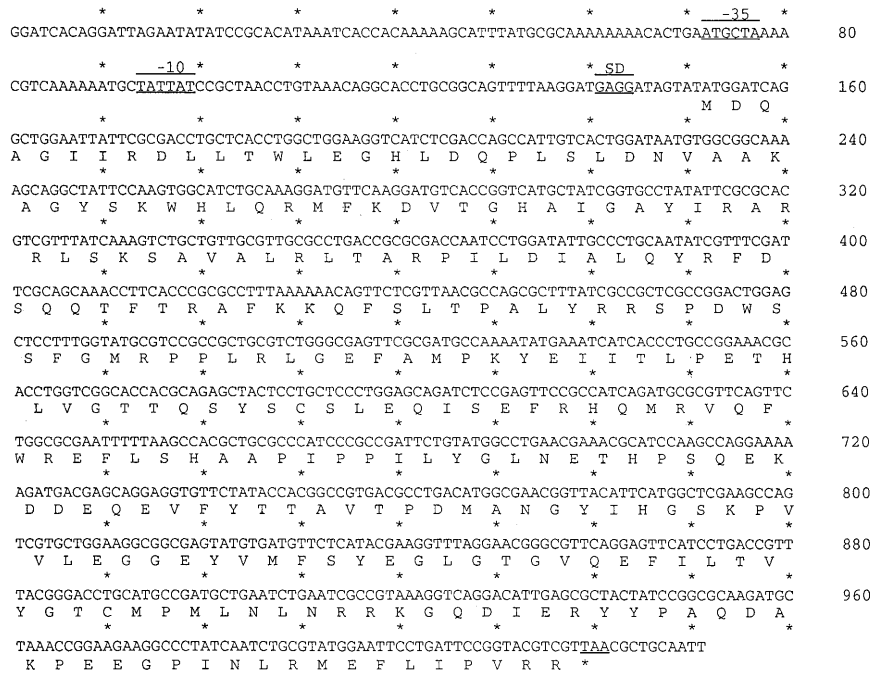

FIG. 2. Nucleotide sequence of the 1,031-bp fragment encoding *robA*(EC1) (base pairs 152 to 1018). A 867-nucleotide open reading frame is preceded by the putative −35 and −10 promoter regions (underlined base pairs 72 to 77 and 94 to 99, respectively). A predicted Shine-Dalgarno (SD) sequence is indicated at base pairs 141 to 144.

sequences from the GenBank database. The strongest match was with protein Rob of *E. coli* (38), with 82.7% amino acid identity (Fig. 3). By analogy, the protein was called RobA (EC1). Rob from *E. coli* has been identified as a DNA binding protein involved in the initiation of replication of the chromosome at *oriC* (38). RobA(EC1) was related to several other proteins, with homology particularly in the *N*-terminal regions, from positions 1 to 106, which contain a helix-turn-helix do-

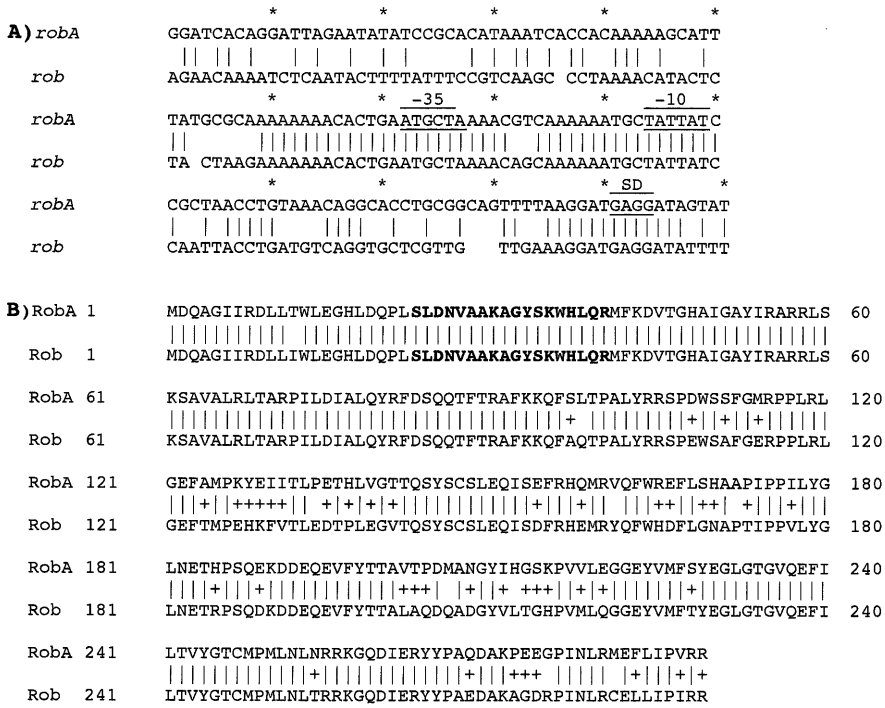

FIG. 3. Alignment of *robA*(EC1) and RobA(EC1) of *E. cloacae* and *rob* and Rob of *E. coli*. (A) Comparison of promoter regions. The −10 and −35 regions and the ribosomal binding site (Shine-Dalgarno sequence [SD]) are underlined. (B) Comparison of predicted amino acid sequences. I, identity; +, similarity. The amino acids of the helix-turn-helix region are indicated in boldface type.

TABLE 3. Effect of *robA*(EC1) on the expression of *ompF* and *ompC*

Strain[a]	Relevant genotype	Plasmid	β-Galactosidase activity[b]
MH225	*ompC-lacZ* operon fusion	pHSS6	452 ± 30
		pAZ902 (*robA*)	414 ± 42
MH513	*ompF-lacZ* operon fusion	pHSS6	363 ± 39
		pAZ902 (*robA*)	319 ± 26
MH610	*ompF-lacZ* protein fusion	pHSS6	637 ± 18
		pAZ902 (*robA*)	56 ± 7
JHC2205	*ompF-lacZ* protein fusion (Δ*micF*)	pHSS6	497 ± 45
		pAZ902 (*robA*)	424 ± 35

[a] Cells were grown overnight at 37°C in the low-osmolarity medium A.
[b] Activities expressed in Miller units (28); values represent an average of at least three independent experiments.

main. The amino acid identities in this region were 97% with Rob, 54% with SoxS, and 47% with MarA from *E. coli* and 48% with PqrA from *Proteus vulgaris* (6, 22, 38, 40) (data not shown). The identities between the 18 amino acids in the helix-turn-helix section were 100, 79, 63, and 68%, respectively (Fig. 3) (data not shown). All five proteins, when either induced or overexpressed, mediated resistance to various unrelated antibiotics (2, 3, 6, 22) (Table 2). Putative −10 (TATT AT) and −35 (ATGCTA) regions for *robA*(EC1) (19) and the ribosomal binding site sequence were identical to those of *rob* from *E. coli*, while an overall homology of 63.9% was observed in a 158-bp stretch immediately upstream of the two genes (38) (Fig. 3).

Effect of RobA(EC1) on the transcription and translation of *ompF* and *ompC*. Plasmid pAZ902 was introduced into the *ompF-lacZ* operon fusion strain *E. coli* MH513, the *ompF-lacZ* protein fusion strain *E. coli* MH610, and the *ompC-lacZ* operon fusion strain *E. coli* MH225. The β-galactosidase activities measured suggested that RobA(EC1) acted preferentially at the level of translation (Table 3).

Effect of RobA on the expression of *ompF* in *micF* deletion strains. To assess the possible contribution of *micF* to the effect of *robA*(EC1) on the translational regulation of *ompF* expression, two *E. coli* strains from which *micF* was deleted, SM3001 and JHC2205, were used. The latter strain harbored an *ompF-lacZ* protein fusion. After introduction of pAZ902 into JHC2205, 85% of the β-galactosidase activity of the control was produced (Table 3), and after introduction into SM3001, examination of outer membranes revealed no decrease in OmpF production (data not shown). The absence of a *robA*(EC1) effect on the expression of *ompF* in the absence of *micF* suggests that it acts via synthesis of the antisense RNA, a situation that has also recently been described for *rob* of *E. coli* (3).

Not all possible functions of RobA(EC1) have been explored. However, since it is closely related (82.7% amino acid identity) to the DNA binding protein Rob (38), it is likely that RobA functions in *E. cloacae* in a manner similar to that of Rob in *E. coli* (3). More specifically and by analogy to what has been observed for MarA, SoxS, and Rob, which are related to the XylS-AraC family of transcription regulators (11), it is also very likely that the activation of *micF* by RobA(EC1), with its helix-turn-helix domain very similar to those of these three proteins, is due to its direct interaction with the *micF* promoter region (3, 5). Implicit in this assumption is the existence of *micF*, or a *micF*-like gene, in *E. cloacae* in which the effect on porins F of RobA produced at levels higher than those produced by the wild type resembles its effect in *E. coli*, in which it reduces OmpF production. A *micF* gene has been reported to occur in several gram-negative species (10) but not in *E. cloacae*. We have found a sequence homologous to *micF* in *E. cloacae* directly by PCR and also by hybridization of *E. cloacae* DNA with the PCR product generated with *micF*-specific primers and *E. coli* target DNA (data not shown), but its nucleotide sequence has not been determined.

With the full physiological role of RobA(EC1) still obscure, it is apparent that *E. cloacae*, like other members of the family *Enterobacteriaceae*, contain an array of genes, such as *romA* (23), *ompX* (39), and *ramA* (13), that can be recruited for a nonspecific response to the stress exerted by various structurally unrelated antibiotics. In this context it is worth mentioning that since MarA, SoxS, and Rob confer similar multidrug resistance phenotypes (3, 6) and since MarA activates the multidrug efflux pump AcrA, which acts in synergy with the OmpF-dependent reduction in permeability, it cannot be excluded that overexpression of Rob also increases multidrug efflux both in *E. coli* and in *E. cloacae* (26, 32).

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