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RamA Is an Alternate Activator of the Multidrug Resistance Cascade in *Enterobacter aerogenes*

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Multidrug resistance (MDR) in *Enterobacter aerogenes* can be mediated by induction of MarA, which is triggered by certain antibiotics and phenolic compounds. In this study, we identified the gene encoding RamA, a 113-amino-acid regulatory protein belonging to the AraC-XylS transcriptional activator family, in the *Enterobacter aerogenes* ATCC 13048 type strain and in a clinical multiresistant isolate. Overexpression of RamA induced an MDR phenotype in drug-susceptible *Escherichia coli* JM109 and *E. aerogenes* ATCC 13048, as demonstrated by 2- to 16-fold-increased resistance to β-lactams, tetracycline, chloramphenicol, and quinolones, a decrease in porin production, and increased production of AcrA, a component of the AcrAB-TolC drug efflux pump. We show that RamA enhances the transcription of the *marRAB* operon but is also able to induce an MDR phenotype in a *mar*-deleted strain. We demonstrate here that RamA is a transcriptional activator of the Mar regulon and is also a self-governing activator of the MDR cascade.

Multidrug resistance (MDR) relates to all bacterial species but has been studied mostly in members of the *Enterobacteriaceae*, particularly in *E. coli* with the study of the *mar* operon (1, 17, 18, 32). The MDR phenotype often associates a decrease in porin synthesis with an increased activity of efflux pumps to restrict the intracellular concentration of various antibiotics, including β-lactams, tetracyclines, chloramphenicol, and quinolones. Resistance results from activation of the chromosomal genes by induction or mutation and by DNA transfer (21). A single drug treatment can lead to cross-resistance to other unrelated antibiotics (16). In clinical isolates which exhibit high resistance to broad-spectrum antibiotics, MDR is the result of enzymatic responses, mutations in the antibiotic target, and modifications in envelope permeability, including porin alteration and induction of drug efflux (7, 20, 23). In such a case, the existence of clinical strains resistant to several structurally unrelated antibiotics contributes to treatment failures in human infections, bacterial dissemination, outbreaks, and changes in patients' flora due to the selective advantage of the microorganism.

The *mar* regulon identified in *E. coli* plays a key role in the expression of the multidrug resistance phenotype. MarA expression induces the synthesis of the *micF* antisense RNA that downregulates the OmpF outer membrane porin, on one hand, and, on the other hand, overproduction of the AcrAB-TolC drug efflux pump (1, 3, 17). Mutations located in the MarR repressor leading to constitutive expression of the *marRAB* operon have been described in fluoroquinolone-resistant isolates of *E. coli* and *Enterobacter cloacae* (20, 25). The Rob and SoxS regulatory proteins are MarA homologues and participate in *E. coli* MDR regulation (1, 16, 28).

Among the emerging resistant bacteria, *E. aerogenes* is now the third leading cause of nosocomial respiratory tract infections (6, 11). A TEM-24-producing clone was the agent of a hospital pandemic (6, 13). Moreover, a concomitant decrease in porin synthesis and quinolone efflux has been reported in several clinical strains (4, 14). In a previous study, rapid modulation of porin synthesis during the course of patient therapy was observed in *E. aerogenes*, suggesting that resistance is mediated by a regulation event rather than a mutation (4). Recently, we described the functional role played in MDR in *E. aerogenes* by the *marRAB* operon, overexpression of which results in major porin decrease and norfloxacin active efflux (9).

In a single-step *Klebsiella pneumoniae* MDR mutant, George et al. characterized a novel genetic system, named *ramA* for "resistance antibiotic multiple" (15). The *K. pneumoniae ramA* locus cloned in *E. coli* elicited a high level of resistance to diverse antibiotics, including chloramphenicol, tetracycline, nalidixic acid, ampicillin, norfloxacin, trimethoprim, and puromycin, a decreased expression of OmpF porin, and the active efflux of chloramphenicol and tetracycline. *ramA* overexpression has been associated with fluoroquinolone resistance in *K. pneumoniae* isolates (30). The *ramA* locus was identified in *Salmonella enterica* serovar Paratyphi B and given the name *rma*, sharing a high percentage of identity (32). Rma was demonstrated to bind to the *mar* operator (32). Surprisingly, an alignment of *K. pneumoniae ramA* with the genome of *E. coli* indicates no homologue of the *ramA* locus.

In the present study, we identified the *ramA* gene of *E. aerogenes*. We demonstrate its involvement in the regulation of outer membrane impermeability and in the active extrusion of intracellular antibiotics. We show that *E. aerogenes* RamA is able to enhance the transcription of the *E. coli marRAB* operon but is also able to induce an MDR genetic cascade in an *E. coli marRAB* deletion background.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features[a]	Source or reference
E. coli		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*)	Laboratory collection
CH164	AG100 Δ*marA zdd-230*::Tn*9*	17
SPC105	MC4100 containing a chromosomal *Pmar*n::*lacZ* fusion at the λ attachment site and a wild-type *mar* locus	10
BW5104	Mu-1 Δ*lac169 creB510 hsdR514*	19
EP663	BW5104 *tolC*::Tn*10*, Tc^r	29
E. aerogenes		
ATCC 13048	Susceptible strain	ATCC
EA27	MDR clinical isolate	29
Plasmids		
pDrive	High-copy-number PCR cloning vector, Amp^r Kn^r	Qiagen
pRC2	pDrive bearing the RaRBS and RaId PCR product to express *ramA* from Plac	This study
pBCSK+	High-copy-number vector; Cm^r	Stratagene
pRC3	EcoRI fragment of pRC2 subcloned into pBCSK+	This study
pmicB21	pICIII bearing a *micF*::*lacZ* fusion	27

[a] Amp^r, ampicillin resistant; Kn^r, kanamycin resistant; Cm^r, chloramphenicol resistant, Tc^r, tetracycline resistant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains were grown in Luria-Bertani (LB) or Mueller-Hinton (MH) broth. Transformed strains were selected on LB agar containing ampicillin at 100 μg/ml, chloramphenicol at 50 μg/ml, or kanamycin at 50 μg/ml.

Antibiotic susceptibility tests. MICs were determined by a standard broth dilution method, as previously described (23). Approximately 10^6 cells were inoculated into 1 ml of MH broth containing twofold serial dilutions of each antibiotic: tetracycline, norfloxacin, cefepime, nalidixic acid, ceftazidime, and chloramphenicol. For the chloramphenicol MIC, phenylalanine arginine β-naphthylamide (PAβN) (Sigma-Aldrich) was added at 26.3 μg/ml; that concentration does not inhibit bacterial growth (22, 24). The results of triplicate tests were read after 18 h at 37°C and correspond to the highest dilution showing complete growth inhibition.

DNA preparation, Genome Walker, primers, and PCR. DNA was prepared with the hexadecyltrimethylammonium bromide (CTAB) method (2). Primers were designed from the sequence of the *E. aerogenes* (RaId and RaRBS) and *K. pneumoniae* (A1, A2, and R2) *ramA* genes with primers A1 (forward; 5′-CCGCTCAGGTGATTGACACT-3′), A2 (reverse; 5′-GGTCTGCTGCGAATCAAGC-3′), R2 (reverse; 5′-GAAGTGGGCAAGAAAACGC-3′), RaId (reverse 5′-CAGATTAAGCCGCAAGAGAGC-3′), and RaRBS (forward; 5′-CGCTAACCGCCAGAGTGGT-3′).

PCR amplifications were performed in a GeneAmp PCR System 2400 thermocycler (Perkin Elmer), with an initial 5-min denaturation at 94°C followed by 30 cycles of denaturation at 94°C for 30 s, a 30-s annealing at 54°C, and a 2-min extension at 72°C. The 30 cycles were followed by a 7-min extension at 72°C. After determination of the partial sequence of the putative *ramA* gene with primers A1 and A2, the unknown 5′-end sequence was amplified with the Universal Genome Walker kit (Clontech Laboratories, Palo Alto, Calif.). Briefly, genomic DNA was digested with EcoRV, DraI, PvuII, StuI, and ScaI. DNA fragments were ligated with a Genome Walker adapter, which had one blunt end and one end with a 5′ overhang. The ligation mixture of the adapter was used as a template for PCR. This PCR was performed with an adapter primer supplied by the manufacturer and the reverse A2 primer to walk along the DNA sequence. The amplification was carried out according to the manufacturer's instructions.

DNA sequencing and data analysis. The sequences of the PCR products were determined with an ABI Prism 377 DNA sequencer with dye fluorescent terminators and the primers used in the initial PCR amplification. Sequence analysis comparison was performed with the lalign program (http://www.ch.embnet.org/software/LALIGN_form.html).

β-Galactosidase assay. The activity of the *E. coli marRAB* and *micF* promoters was measured by assaying the β-galactosidase activity of a chromosomal *mar*::*lacZ* operon fusion and a plasmid *micF*::*lacZ* fusion as described by Miller (26a).

Measurement of chloramphenicol accumulation. Exponential-phase bacteria grown in LB broth were removed by centrifugation and washed once in 50 mM sodium phosphate buffer (SPB), pH 7. Pellets were suspended in the same buffer to a density of 2×10^{10} CFU ml^{-1} and kept at 37°C for no more than 30 min before use. To deenergize the bacteria, 50 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Sigma-Aldrich) was added 10 min before the radiolabeled chloramphenicol. Assays were initiated by adding [^{14}C]chloramphenicol (10 μg/ml, 10^{10} cpm/ml; a gift from Aventis Hoescht Marrion Roussel Laboratories). Samples (100 μl) were removed at set intervals and immediately filtered through 0.45-μm Whatman GF/C filters presoaked in SPB and then washed twice with 5 ml of cold SPB. The filters were dried, and the radioactivity was determined in a Beckman scintillation spectrophotometer. Positive and negative control samples were run under identical conditions (23, 24). The accumulation experiments were performed in duplicate.

SDS-PAGE and immunodetection of porins and AcrA. Exponential-phase bacteria grown in LB broth were pelleted and solubilized in boiling buffer at 96°C. Samples (an amount corresponding to 0.02 optical density units at 600 nm) were loaded on sodium dodecyl sulfate (SDS)-polyacrylamide gels (10% polyacrylamide, 0.1% SDS) for SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (8) and then electrotransferred to nitrocellulose membranes. An initial saturating step with Tris-buffered sodium (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 8) containing 10% skim milk powder was carried out overnight at 4°C. The nitrocellulose membranes were then incubated in TBS containing 10% skim milk powder and 0.2% Triton X-100 for 2 h at room temperature in the presence of polyclonal antibodies directed against the internal porin L3 loop or against AcrA. The polyclonal antibody F4 was prepared against a peptide located within the internal porin L3 loop and recognized enterobacterial porins as previously described (12). The polyclonal anti-AcrA antibody has been previously described (29). After four washes in the same buffer, detection was performed with alkaline phosphatase-conjugated Affinity-Pure goat anti-rabbit immunoglobulin G antibodies (Jackson ImmunoResearch).

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been submitted to the EMBL database and given accession no AJ404625.

RESULTS

Identification of *ramA* in *E. aerogenes*. The *E. aerogenes ramA* gene was PCR amplified with primers A1 and A2, chosen from the *K. pneumoniae ramA* sequence, and the reverse R2 primer, chosen in the 3′ flanking region surrounding the *K. pneumoniae ramA* gene. PCR assays were performed on the genomic DNA of *E. aerogenes* ATCC 13048 (type strain) and

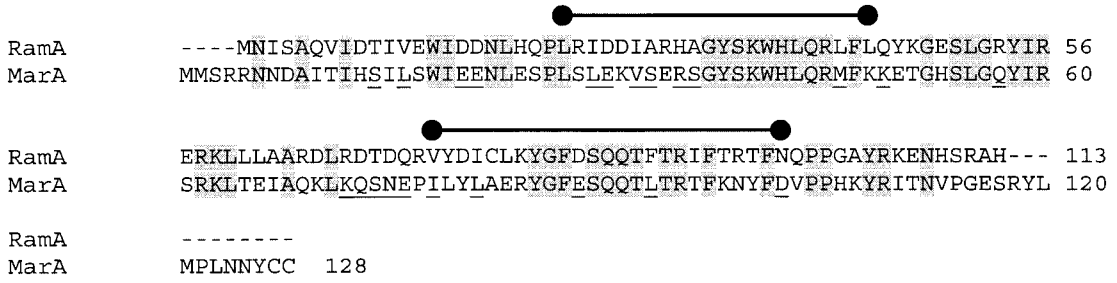

FIG. 1. Multiple-sequence alignment of RamA and MarA of *E. aerogenes*. Sequences were aligned by use of the Lalign program. The locations of the two helix-turn-helix motifs are marked by overlining, identical residues are shaded, and conservative replacements are underlined.

on the MDR clinical isolate EA27 (23, 29). For both strains with primers A1 and A2, an identical 339-bp sequence encoding a 113-amino-acid putative protein was obtained. The sequence of the promoter region was obtained by the genome walker method on *E. aerogenes* ATCC 13048 DNA restricted by ScaI and amplified with the A2 primer and the Ap2 primer supplied by the manufacturer. An identical *ramA* product was also PCR amplified with primers A1 and A2 in all 39 *E. aerogenes* clinical isolates tested (data not shown). Alignment of the predicted amino acid sequence of RamA from *E. aerogenes* with those of the two known homologues in *S. enterica* and *K. pneumoniae* exhibited high interspecific conservation. The putative sequence of RamA of *E. aerogenes* showed greater amino acid similarity with RamA of *K. pneumoniae* (98%) than with Rma of *S. enterica* (92%) (lalign program).

RamA sequence analysis and putative marbox. The RamA and MarA alignment shows that amino acids in their helix-turn-helix DNA binding motif are mostly conserved, suggesting that the two proteins could regulate the same target genes (Fig. 1). The transcriptional activator MarA binds as a monomer to a 20-bp degenerate asymmetric recognition sequence named the marbox (Fig. 2) (26). As MarA is able to autoregulate its expression, we looked for the presence of a marbox in the promoter region of *ramA*. From the consensus sequence of the *E. coli* marbox and the putative MarA binding site of the *E. aerogenes mar* operon, we identified a putative marbox in the *ramA* promoter region (Fig. 2). This putative marbox is in a backward orientation and would belong to class I of the MarA-activated promoters (26).

***ramA* overexpression induces multidrug resistance in *E. coli* and in *E. aerogenes*.** The 447-bp PCR product obtained by *ramA* amplification with the RaRBS and RaId primers, including the whole open reading frame and the ribosome binding site, was cloned into plasmid pDRIVE to generate pRC2. To test the hypothesis that RamA acts as an MDR activator, we introduced pRC2 into *E. coli* JM109 and into *E. aerogenes* ATCC 13048. Both transformants presented a 2- to 16-fold increase in MICs of the antibiotics tested (Table 2). Thus, *ramA* overexpression confers an MDR phenotype on *E. coli* and *E. aerogenes*. The MICs for chloramphenicol were evaluated in the presence of PAβN, a nonspecific efflux pump inhibitor (22, 24). In the presence of PAβN (26.3 μg/ml), the MIC of chloramphenicol for JM109(pRC2) decreased eightfold, from 64 to 8 μg/ml, indicating the activity of an efflux system in the transformed strains.

Consequently, to evaluate the importance of the efflux mechanism in the resistance mediated by RamA, pRC2 was introduced into an *E. coli tolC* null mutant strain. Compared to the BW5104 parental strain, a decrease in the MICs for chloramphenicol (eightfold) and nalidixic acid (16-fold) was observed in the *tolC* mutant (EP663), showing that efflux mediated by TolC is involved in the resistance to these antibiotics (Table 2). In the absence of TolC, RamA overproduction did not increase chloramphenicol and nalidixic acid resistance levels, indicating that *ramA*-mediated efflux requires TolC. For β-lactams such as cefepime and ceftazidime, which work by decreasing permeability, inactivation of *tolC* had no effect on the resistance level even when *ramA* was overexpressed.

Evidence of active efflux of chloramphenicol in *E. coli* expressing *ramA*. The increase in the MICs for chloramphenicol and tetracycline in the strain containing pRC2 suggested the presence of an efflux pump (Table 2). To examine this mechanism, the intracellular accumulation of radiolabeled chloramphenicol was studied in *E. coli* JM109(pRC2). A severe decrease in the intracellular accumulation of [^{14}C]chloramphenicol was measured in the presence of pRC2, with a reduction of about fivefold compared to JM109 (Fig. 3). The addition of CCCP, which collapses the membrane energy and blocks the energy-dependent efflux pump, induced a noticeable increase in the intracellular level of [^{14}C]chloramphenicol in JM109(pRC2) (23, 24). CCCP partially restored a level of intracellular chloramphenicol similar to that in the wild-type strain. These results suggest that RamA production from pRC2 is able to induce the expression of an active efflux pump.

***ramA* overexpression in *E. coli* and in *E. aerogenes* causes AcrA increase and porin decrease.** Rma was shown to induce MDR by porin downregulation associated with a TolC increase, evidenced by Western blotting, suggesting the presence of efflux (32). In order to characterize the *ramA* MDR activation pathway, immunodetection of porins and AcrA was performed with the F4 antipeptide, directed against the porin L3 loop on *E. coli* JM109(pRC2) and *E. aerogenes* ATCC 13048(pRC2) and with AcrA antibodies on *E. aerogenes* ATCC 13048(pRC2). RamA overexpression in *E. coli* JM109 and in *E. aerogenes* ATCC 13048 harboring pRC2 induced a noticeable decrease in production of the major porin. Conjointly, we observed overexpression of AcrA, the periplasmic component of the AcrAB-TolC efflux pump in *E. aerogenes* ATCC 13048(pRC2) (Fig. 4).

RamA induces an MDR phenotype in a *marRAB* deletion background. The precise role of RamA in the MDR regulation cascade remains quite obscure. In order to define whether

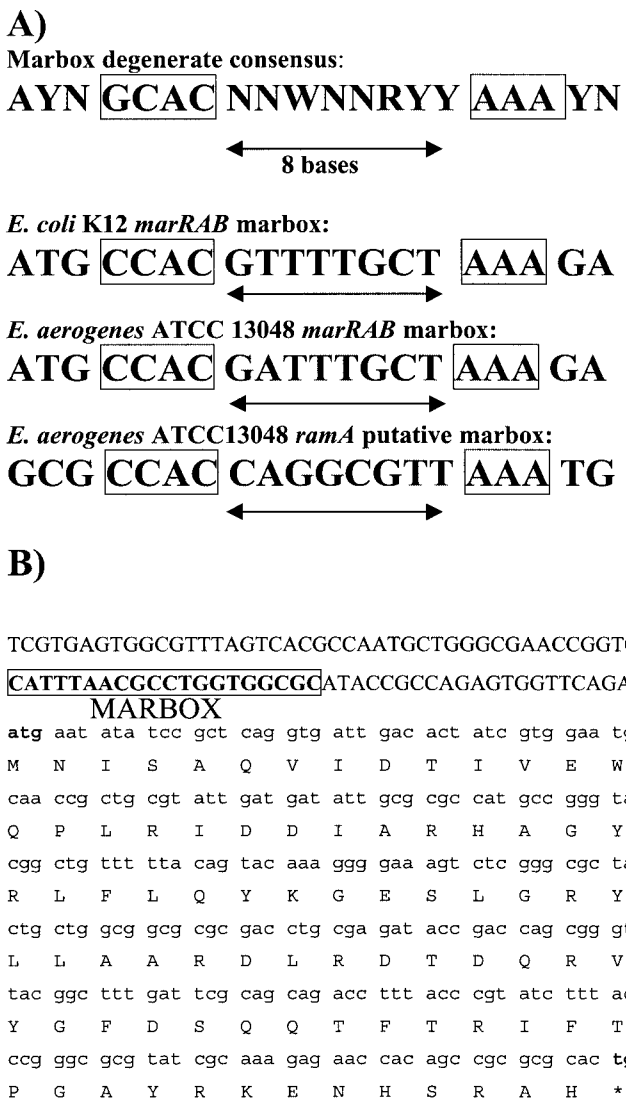

FIG. 2. (A) Alignment of the marboxes of the *E. coli* and *E. aerogenes mar* operons and of *E. aerogenes ramA* with the consensus (N, any base; W, A or T; R, A or G; Y, C or T). (B) Position of the *E. aerogenes* putative *ramA* marbox on the nucleotide sequence of *ramA*; the start and stop codons are in bold.

RamA is able to induce an MDR response by a *marRAB* operon independent pathway, the *E. coli* CH164 Δ*mar* strain was transformed with pRC2. Measures of antibiotics susceptibility and immunodetection of porins and AcrA were performed. The transformed strain exhibited increased MICs to structurally unrelated antibiotics (Table 2) and a porin production decrease associated with an increase in AcrA synthesis (Fig. 4).

The MDR phenotype in CH164(pRC2) was similar to that observed in JM109(pRC2). Thus, RamA is able to induce an MDR cascade independently of the *mar* operon.

RamA effect on *marRAB* operon and *micF* RNA. Yassien et al. previously reported that a MalE-Rma hybrid is able to bind to the *E. coli mar* operator (32). To determine whether RamA can regulate *mar* operon transcription, we investigated the effect of RamA on a *mar*::*lacZ* chromosomal fusion in *E. coli*. *E. coli* SPC105 was transformed with pRC3 bearing *ramA*, and

TABLE 2. Effect of RamA expression

Strain	MIC[a] (μg/ml)					
	CHL	TET	NAL	NOR	CAZ	FEP
E. coli						
JM109	8	1	64	0.5	0.5	0.047
JM109(pRC2)	64	16	128	2	0.5	0.38
CH164	8	4	8	0.5	2	0.25
CH164(pRC2)	32	16	32	1	4	2
BW5104	16	4	4	0.25	0.25	0.016
BW5104(pRC2)	64	16	16	0.5	1	0.094
EP663	2	R	0.25	0.5	0.125	0.016
EP663 (pRC2)	2	R	0.25	0.06	1	0.094
E. aerogenes						
ATCC 13048	8	4	8	0.5	2	0.25
ATCC 13048(pRC2)	32	16	32	1	4	2

[a] CHL, chloramphenicol; TET, tetracycline; NAL, nalidixic acid; NOR, norfloxacin; CAZ, ceftazidime; FEP, cefepime; R, resistant.

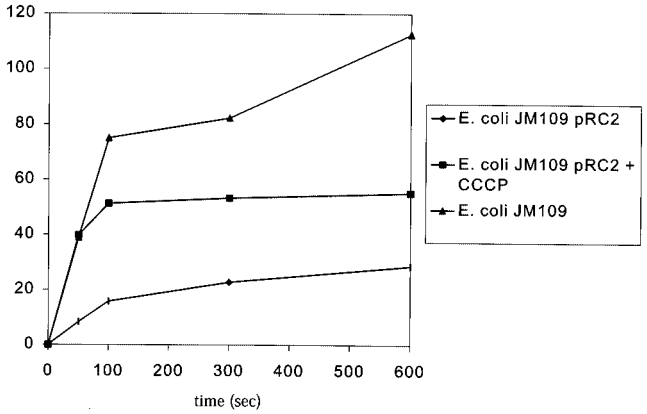

FIG. 3. Accumulation of [^{14}C]chloramphenicol in *E. coli* JM109 (▲), JM109(pRC2) (♦), and JM109(pRC2) in the presence of CCCP (■).

β-galactosidase assays were performed. Since pRC3 encodes chloramphenicol resistance and chloramphenicol is an inducer of the *mar* operon expression (1), experiments were carried out in the absence of chloramphenicol, and the presence of pRC3 was verified after each assay. The results indicate that in the presence of pRC3, the β-galactosidase activity of the *mar*::*lacZ* strain was increased fourfold. The β-galactosidase activities of *E. coli* strains SPC105 *mar*::*lacZ*, SPC105(pRC3), JM109, JM109(pmicB21), and JM109(pmicB21, pRC3) were 480 ± 57, 1,950 ± 132, 80 ± 6, 530 ± 50, and 1,880 ± 73 Miller units, respectively (mean ± standard deviation).

To assay whether RamA affected *micF* transcription, JM109 was cotransformed with pmicB21 bearing a *micF*::*lacZ* fusion and with pRC3. The β-galactosidase activity in JM109 (pmicB21) was increased 3.5-fold in presence of pRC3 (see above). *micF* activation may be due to direct activation by RamA and/or indirect activation through the *mar* operon. These results suggest that RamA is able to induce transcription of both the *mar* operon and the anti-sense *micF* RNA in *E. coli*.

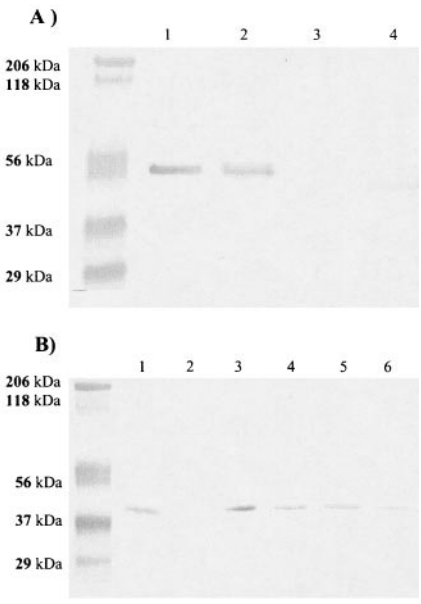

FIG. 4. Immunoblots of whole-membrane extracts with antibodies raised against *E. aerogenes* AcrA (A) and against porins (F4 antibodies) (B). (A) Lane 1, *E. aerogenes* ATCC 13048; lane 2, *E. aerogenes* ATCC 13048(pRC2); lane 3, *E. coli* CH164; lane 4, *E. coli* CH164 (pRC2). Lane 1, *E. coli* JM109; lane 2, *E. coli* JM109(pRC2); lane 3, *E. aerogenes* ATCC 13048; lane 4, *E. aerogenes* ATCC 13048(pRC2); lane 5, *E. coli* CH164; lane 6, *E. coli* CH164(pRC2).

DISCUSSION

In this study, we characterized the *E. aerogenes ramA* gene and its role in the induction of the MDR response. RamA overexpression in the susceptible *E. coli* JM109 and in *E. aerogenes* ATCC 13048 strains induces an MDR phenotype, as evidenced by increases in the MICs of structurally unrelated antibiotics. We showed that RamA induces a significant reduction in the amount of intracellular radiolabeled chloramphenicol. Strains expressing RamA are less permeable due to a loss of the major porins, and in addition they produce larger amounts of the AcrA periplasmic linker protein, involved in the AcrAB-TolC efflux pump.

RamA belongs to the AraC-XylS regulator family. In this broad family, RamA is very close to a group of MDR regulators, including MarA, SoxS, and Rob. RamA and MarA from *E. aerogenes* share 45% identity, and the amino acids of the two DNA binding motifs essential to the regulatory function of MarA are well conserved in RamA. It is tempting to speculate that RamA and MarA recognize an overlapping set of operator sequences. This idea is supported by the ability of strains expressing RamA to overexpress *acrA* and *micF*, two target genes of MarA. Moreover, the presence of a putative marbox in the *ramA* promoter has been detected. This putative marbox is very well conserved, according to the consensus (26). MarA and also RamA could therefore regulate the transcription of *ramA*.

The transcriptional activator MarA autoregulates the expression of the *marRAB* operon via its binding to a marbox on its own operator (1). Using a *lac* reporter gene fused to the *mar* promoter, we showed that RamA overproduction is able to enhance expression of the *E. coli mar* operon. As the *marRAB* operons of *E. coli* and *E. aerogenes* share a high percentage of identity (9), we propose that RamA is able to modulate *E. aerogenes marRAB* operon transcription. In contrast to *E. coli*, in which no *ramA* homologue have been found in the two genomes sequenced (strains K-12 and O157:H7), *E. aerogenes* possesses two MDR transcriptional activators, MarA and RamA.

The major finding of this study is that constitutive expression of RamA results in an MDR phenotype even in the absence of the *mar* locus. Indeed, *E. coli* CH164 Δ*mar* transformed with pRC2 exhibited an increase in the MICs of unrelated antibiotics, a loss of porins, and an increase in AcrA expression. The RamA-induced phenotype is very similar to that induced by MarA, but RamA is able to activate the MDR cascade independently of MarA. It is important to notice that these results indicate that *E. aerogenes* is able to regulate the *marRAB* operon by two processes: the chemical effector way with salicylate (9) and the genetic regulator way with RamA, described here. Thus, RamA can promote MDR by direct activation of

acrAB and *micF* and indirect activation through MarA. The duplication of MDR activation pathways may explain the rapid and efficient adaptive response of *E. aerogenes* during antibiotic treatment and the emergence of MDR strains in vivo (4, 5, 14). It is now important to define the conditions of *ramA* expression and the benefit to bacteria of possessing two genes, *ramA* and *marA*, with a similar function. Therefore, we need to study the relationship between the MarA and RamA genetic systems to decipher their individual involvement in MDR.

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