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Molecular Cloning and Characterization of the *Salmonella enterica* Serovar Paratyphi B *rma* Gene, Which Confers Multiple Drug Resistance in *Escherichia coli*

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A genomic library from a strain of *Salmonella enterica* serovar Paratyphi B that exhibits multiple drug resistance (MDR) was constructed in *Escherichia coli*. Two of the recombinant plasmids, pNOR5 and pNOR5, conferred resistance only to fluoroquinolones in *E. coli*, whereas the third, pNCTR4, conferred the MDR phenotype. Sequence and subcloning analysis showed that it is the presence of RecA on the first two plasmids which confers resistance to fluoroquinolones in *E. coli*. A similar analysis established that the MDR phenotype conferred by pNCTR4 is due to a gene, *rma* (resistance to multiple antibiotics), which encodes a 13.5-kDa polypeptide. The derived sequence for Rma exhibits a high degree of similarity to those of a group of MarA-like activators that confer MDR in *E. coli*. A MalE-Rma fusion protein was purified to near homogeneity and was shown to interact with a DNA fragment carrying a MarA operator sequence. Furthermore, overexpression of *rma* in *E. coli* caused changes in the outer membrane protein profile that were similar to those reported for MarA. These results suggest that Rma might act as a transcriptional activator of the *marA* regulon.

The emergence of clinical isolates of salmonellae that exhibit multiple drug resistance (MDR) represents an increasing threat to human health. While the use of antibiotics in livestock feed is considered a primary cause of this phenomenon, the extensive use of fluoroquinolones in the treatment of typhoid also led to the frequent isolation of typhoid strains that exhibit MDR in numerous outbreaks in developing countries in Africa and south Asia (26, 32). Recently, the first recognized outbreak of fluoroquinolone-resistant *Salmonella enterica* serovar Paratyphi infection in the United States was reported in two nursing homes and one hospital in Oregon (23).

The molecular mechanisms of MDR have been studied extensively in *Escherichia coli*. These include the induction of efflux pumps to promote extrusion (the *acrRAB* and *emrRAB* loci) as well as the down regulation of outer membrane porins (e.g., OmpF) to reduce the inflow of a variety of structurally unrelated antibiotics (16, 18, 22). The *marRAB* (5) and *soxRS* (21, 35) systems exert overlapping effects on the regulation of efflux pumps and porin synthesis in *E. coli* via a sophisticated control circuit (18). While the natural stimulants for the *mar* and *sox* systems overlap and some of their derivatives are structurally related, they contribute independently to the control network of MDR through direct interactions with the affected operons. A recent paper by Barbosa and Levy (2) reported on the differential expressions of over 60 chromosomal genes in the MarA regulon of *E. coli*. RamA of *Klebsiella pneumoniae* and *Enterobacter cloacae* and PqrA of *Proteus vulgaris*, which have moderate sequence similarities to the *E. coli* MarA protein, have also been reported to confer MDR when they are overexpressed in *E. coli* (11, 13, 15).

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Understanding of the molecular basis of MDR is important for the design and use of antimicrobial agents. One of the laboratories collaborating on the present study has previously reported on the isolation of a derivative of *S. enterica* serovar Paratyphi B that exhibits the MDR phenotype (8). We initiated the present study in order to identify the genetic determinants of this resistance. The study described in the present paper indicates that the *rma* (resistance to multiple antibiotics) gene of serovar Paratyphi B confers the MDR phenotype in *E. coli*. We report on an analysis of the outer membrane proteins in a recombinant strain of *E. coli* which overexpresses a MalE-Rma fusion protein as well as the results of gel retardation assays with the purified fusion protein. The results indicate that the Rma protein is a new member of the MarA-SoxS family of transcriptional regulators.

MATERIALS AND METHODS

Strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. Unless otherwise indicated, cultures were grown in Luria-Bertani (LB) enriched medium under aerobic condition at 37°C with the following supplements, as required: ampicillin at 50 μg/ml and norfloxacin at 2 μg/ml. Cell growth was monitored optically at 600 nm.

Construction of a shotgun genomic library. Chromosomal DNA was prepared from a derivative of *S. enterica* serovar Paratyphi B that exhibits MDR with an AquaPure genomic DNA isolation kit (Bio-Rad Laboratories). The DNA was partially digested with *Sau*3AI, and fragments of 1 to 10 kb were separated by electrophoresis on 0.5% agarose and purified with a QIAEX II gel extraction kit (Qiagen). The vector plasmid, pUC19, was treated with restriction endonuclease *Bam*HI and was subsequently dephosphorylated with bacterial alkaline phosphatase. The ligation mixture was incubated with T4 DNA ligase (Boehringer Mannheim GmbH, Mannheim, Germany) overnight at 14°C. For transformation, competent *E. coli* DH5α cells were prepared by the method of Inoue et al. (12).

Drug susceptibility testing. The MICs of the antimicrobial agents were determined by the broth microdilution technique (19). Twofold serial dilutions of antimicrobial agents in 100 μl of antibiotic medium 3 with an inoculum of 10^3 to 10^4 CFU of logarithmically grown cells were prepared in 96-well microtiter plates. The range of antibiotic concentrations tested was 0 to 128 μg/ml. The

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
E coli DH5α	*supE44 lacU169 (80lacM15) hsdR1 recA1 endA1 gyrA96 thi-1 relA1*	New England BioLabs
S. enterica serovar Paratyphi B	Wild-type strain; a clinical isolate	8
S. enterica serovar Paratyphi B M95	Mutant strain with MDR	8
Plasmids		
pUC19	Multicopy vector, AMP^r	New England BioLabs
pNCTR4	NOR^r CHL^r TET^r GEN^s	This study
pGEM-T	PCR cloning vector	Promega
pNCTR44	NOR^r CHL^r TET^r GEN^s	This study
pNCTR42	NOR^r CHL^r TET^r GEN^s	This study
pNOR5	NOR^r	This study
pNOR6	NOR^r	This study
pNOR7	*recA* subclone, NOR^r	This study
pMal-c2X	Purification and expression vector	New England BioLabs
pMal-Rma	MalE-Rma protein fusion	This study

[a] NOR, norfloxacin; CHL, chloramphenicol; TET, tetracycline, GEN, gentamicin; AMP, ampicillin.

MIC was defined as the lowest concentration of the antimicrobial agent that inhibits visible growth after 18 to 24 h of incubation at 37°C. The MICs reported here represent the means for quadruplicate experiments.

Preparation of outer membrane proteins and their analysis by polyacrylamide gel electrophoresis (PAGE). Crude outer membrane proteins were prepared by the procedure described by Spratt (29). Cells were grown in 1 liter of LB broth at 37°C with vigorous aeration and were harvested in the mid-log phase to the early stationary growth phase. After the cells were cooled on ice, they were centrifuged at 6,000 × *g* for 6 min in a GSA rotor and were resuspended in 25 ml of ice-cold 10 mM sodium phosphate (pH 7.0). The cells were disrupted by passage twice through a French pressure cell (Sim-Aminco; Spectronic Unicam, Rochester, N.Y.) at 15,000 lb/in^2. Large cell debris and unbroken cells were removed by centrifugation at 8,000 × *g* for 20 min at 4°C in the SS-34 rotor of a Sorvall RC2B centrifuge. The cell membranes were pelleted out of the supernatant by centrifugation at 100,000 × *g* for 40 min at 4°C in the Ti 70 rotor of an XL-90 Beckman ultracentrifuge. The membranes were suspended in 6 ml of ice-cold phosphate buffer, washed twice by centrifugation at 100,000 × *g*, and finally stored in the same buffer at a concentration of 40 mg/ml at −80°C.

The crude outer membrane proteins were subjected to further purification by the procedure described by Sawai et al. (28). The crude outer membrane fraction obtained from 1 liter of bacterial culture was suspended in 3 ml of 10 mM phosphate (pH 7.0) containing 2.0% sodium lauroyl sarcosinate (Sigma), and the mixture was incubated at 20°C for 10 min. The suspension was then centrifuged at 100,000 × *g* for 40 min at 10°C in a 50 Ti rotor (Beckman-Coulter). The pellet was resuspended, washed twice with 1% lauroyl sarcosine in phosphate buffer, and then used as the purified outer membrane preparation. The concentration of the protein was measured by a protein assay (Bio-Rad Laboratories, Hercules, Calif.).

Sodium dodecyl sulfate (SDS)-PAGE of the outer membranes was performed in a 10% acrylamide gel by the method of Sambrook et al. (27). The purified outer membrane protein, solubilized in the same volume of 2× SDS gel-loading buffer (1× buffer is composed of 50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), was heated in boiling water for 2 min before application. The gel was stained with Coomassie brilliant blue G (Sigma).

Western blot analysis of TolC. The level of TolC expression was determined with antiserum specific for the TolC protein and purified outer membranes. Equal amounts (30 μg each) of outer membrane proteins were subjected to separation by SDS-PAGE on a 10% gel. The gel was then equilibrated in transfer buffer containing 10 mM Tris base, 200 mM glycine, and 10% methanol for 5 min before it was electroblotted onto a polyvinylidene difluoride membrane (Applied Biosystems). The standard protocol for Western blotting was followed (31), and the immunocomplex was detected by the presence of alkaline phosphatase-conjugated secondary antibody and the chromogenic substates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Expression and purification of MalE-Rma fusion protein. The pMAL protein fusion system (New England BioLabs) was used for expression of the MalE-Rma fusion protein. The structural gene of *rma* was amplified by PCR with *Pfu* polymerase (Promega) and two oligonucleotide primers whose sequences were specific for regions flanking *rma*. The purified PCR product was ligated to *Xmn*I-digested vector pMAL-c2X so that the first methionine codon was fused in frame to the C terminus of a modified MalE without the signal peptide. After introduction of the plasmid into competent *E. coli* DH5α cells, successful transformants were selected by the protocol suggested by the manufacturer. The resulting recombinant plasmid, pMAL-Rma, was confirmed by restriction analysis and DNA sequencing. Synthesis of the MalE-Rma protein in the recombinant *E. coli* strain harboring pMAL-Rma was induced by the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Following growth and induction, the cells were harvested by centrifugation at 5,000 × *g* at 4°C. The cells were resuspended in 50 mM Tris-HCl (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride and were disrupted as described above. Ruptured cells were centrifuged at 12,000 × *g* for 10 min at 4°C to eliminate cell debris and unbroken cells. The supernatant was loaded at a flow rate of 1.0 ml/min onto a 10-ml heparin column (Pharmacia), which was preequilibrated with 50 mM Tris-HCl (pH 8.0) at 4°C. Proteins were eluted with a linear gradient of KCl in the same buffer. The 55-kDa MalE-Rma fusion protein, monitored by SDS-PAGE and mobility shift assays, was eluted at 0.2 M KCl. Fractions containing the MalE-Rma fusion protein were combined and passed onto a 20-ml amylose affinity column (New England BioLabs). The MalE-Rma fusion protein was released from the column by elution with 50 mM Tris-HCl (pH 7.5)–1 mM EDTA–10 mM maltose. The protein concentration was determined by the method of Bradford (3).

Mobility shift assays. A 26-bp fragment of double-stranded DNA containing the MarA operator sequence, 5′-GGGATTTAGCAAACGTGGCATCGG-3′, was generated from the annealing of two oligonucleotide primers with complementary sequences. For use as a probe in mobility shift assays, the 26-bp double-stranded DNA fragment was labeled with [γ-^{32}P]ATP by use of the DNA polymerase Klenow fragment, and the excess labeled nucleotide was eliminated with Sephadex G-25.

The binding reaction mixture (20 μl) contained various concentrations of the purified MalE-Rma fusion protein in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA in the presence of 0.2 μg of nonspecific sheared chromosomal DNA of *Bacillus subtilis.* The reaction mixtures were incubated at room temperature for 15 min before they were loaded onto a 10% native polyacrylamide gel equilibrated with running buffer containing 40 mM Tris-acetate and 1 mM EDTA. The gels were prerun for 30 min before they were loaded. After the gels were loaded, they were run at 24 mA until the bromophenol blue dye front had migrated at least 50% of the gel length. The gels were transferred to a piece of filter paper, dried, and exposed to a phosphorimager plate (Fuji) for image analysis.

Nucleotide sequence accession number. The nucleotide sequence was determined with an ABI Prism 377 DNA sequencer. The sequence of *rma* and its flanking regions has been deposited in the GenBank database under accession number AF411103.

RESULTS

Cloning of *S. enterica* serovar Paratyphi B MDR genetic determinants. A previous report described the isolation and characterization of a norfloxacin-resistant derivative of *S. en-*

TABLE 2. Effect of Rma expression on antibiotic susceptibility

Strain	MIC (μg/ml)[a]			
	Nor-floxacin	Chloram-phenicol	Tetra-cycline	Genta-micin
E. coli	0.05	2.0	0.5	1.0
S. enterica serovar Paratyphi	0.1	2.0	0.5	1.0
S. enterica serovar Paratyphi M95	8.0	16.0	4.0	1.0
E. coli/pNOR5	16.0	2.0	0.5	1.0
E. coli/pNCTR4	8.0	16.0	4.0	1.0
E. coli/pMal-c2X	0.05	2.0	0.5	1.0
E. coli/pMal-Rma	8.0	16.0	4.0	1.0

[a] Determined by the broth microdilution technique (19). MICs represent the means for quadruplicate experiments.

terica serovar Paratyphi B that exhibited an MDR phenotype (8). The MICs of a number of antibiotics were determined for mutant strain M95 (Table 2). The results show that the MICs of norfloxacin, tetracycline, and chloramphenicol for M95 are significantly higher than those for the wild-type strain, while the MIC of gentamicin was unchanged.

To identify the genetic determinants for the MDR phenotype of *S. enterica* serovar Paratyphi B M95, a shotgun genomic library of this mutant strain was constructed by use of plasmid pUC19 as the cloning vector. The resulting shotgun library cocktail was introduced into *E. coli* DH5α by transformation, and transformants were selected on LB agar plates containing 100 μg of ampicillin per ml and 2 μg of norfloxacin per ml. Three clones were obtained from this experiment, and the presence of recombinant plasmids in these clones was confirmed by restriction enzyme digestion. The MICs of norfloxacin, tetracycline, chloramphenicol, and gentamicin for the three *E. coli* recombinants and parent strain *E. coli* DH5α were determined. Two *E. coli* recombinants, carrying pNOR5 and pNOR6, respectively, exhibited a phenotype of resistance to norfloxacin only. Only one *E. coli* recombinant, harboring plasmid pNCTR4, displayed the MDR phenotype, with the MICs for the transformant being similar to those for serovar Paratyphi B M95 (Table 2).

Overexpression of RecA causes norfloxacin-specific resistance in *E. coli* DH5α. As described above, three recombinant strains of *E. coli* DH5α showing specific resistance to norfloxacin were identified from shotgun cloning experiments. Sequence analysis indicated that two of the three plasmids, pNOR5 and pNOR6, contained the *recA* gene. Earlier reports have indicated that the RecA protein plays a role in the repair of DNA damage following exposure to quinolones (14, 24, 33). Accordingly, the *recA* gene of *S. enterica* serovar Paratyphi B along with its putative ribosomal binding site was amplified by PCR and cloned into pUC19. When the resulting plasmid, pNOR7, was introduced into *E. coli* DH5α, which possesses the *recA1* mutation, the resulting recombinant strain retained resistance to norfloxacin. These results show that the *recA* gene alone confers resistance to norfloxacin in *E. coli*.

Identification of the *rma* gene conferring MDR in *E. coli*. The 14-kb chromosomal insert of pNCTR4 was digested with restriction endonucleases *Eco*RI and *Hin*dIII, and the resulting three restriction fragments were cloned separately into pUC19 for subcloning analysis. The MICs for *E. coli* recombinants harboring the resultant plasmids (pNCTR41, pNCTR42, and pNCTR43) were determined. The results (Table 2) revealed that plasmid pNCTR42 harbors the gene that confers MDR in *E. coli*. The nucleotide sequence of the 5.6-kb *Eco*RI insert of pNCTR42 was determined and was subsequently analyzed by a search for homology with the sequences in the nonredundant database as well as the unfinished genomic sequences of *S. enterica* serovar Paratyphi A and *S. enterica* serovar Typhimurium at the National Center for Biotechnology Information (NCBI) with the BLAST program. The results of the sequence comparison indicate that the 5.6-kb insert of pNCTR42 is most likely the ligation product of two noncontiguous chromosomal fragments. Importantly, the chromosomal insert in pNCTR42 contains an intact open reading frame (ORF) that appeared to be a particularly promising candidate as a genetic determinant of MDR on the basis of a comparison of its sequence with the sequences in the protein database at NCBI. This ORF encodes a putative polypeptide of 113 amino acids (13.5 kDa) which exhibits 75 to 88% sequence identity to a group of transcriptional activators that have been reported to confer MDR (11, 13, 15): RamA of *K. pneumoniae* (GenBank accession number Q48413), *Enterobacter aerogenes* (GenBank accession number CAB95659), and *E. cloacae* (GenBank accession number P55922). The amino acid sequence of this ORF possesses a moderate 38% sequence identity to those of MarA (GenBank accession number L06966) and SoxS (GenBank accession number M60111) of *E. coli* (Fig. 1).

The sequence containing this ORF along with the preceding putative ribosomal binding site was amplified from pNCTR42 by PCR with *Taq* DNA polymerase and two flanking oligonu-

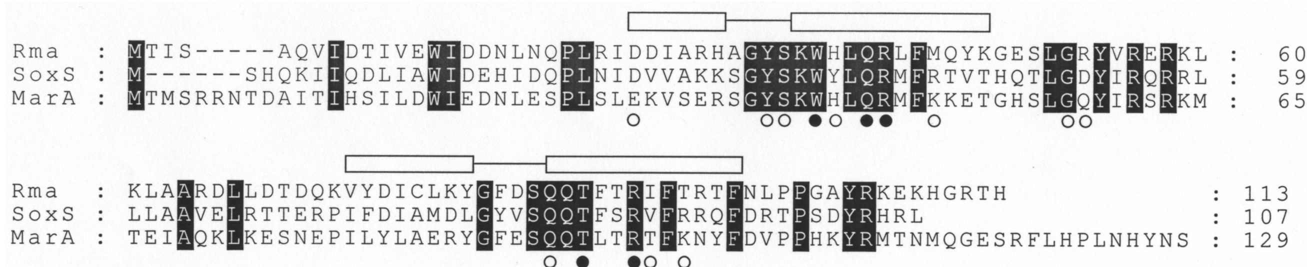

FIG. 1. Multiple-sequence alignment of Rma, SoxS, and MarA. Sequences were aligned by use of the Clustal W program (30). Residues that are conserved among these proteins are shaded. The locations of two helix-turn-helix motifs of MarA are marked with open boxes (helices) connected by lines (turns). Black circles, MarA residues that are involved in determination of sequence specificity; open circles, residues in contact with the phosphate backbone of the operator DNA (25).

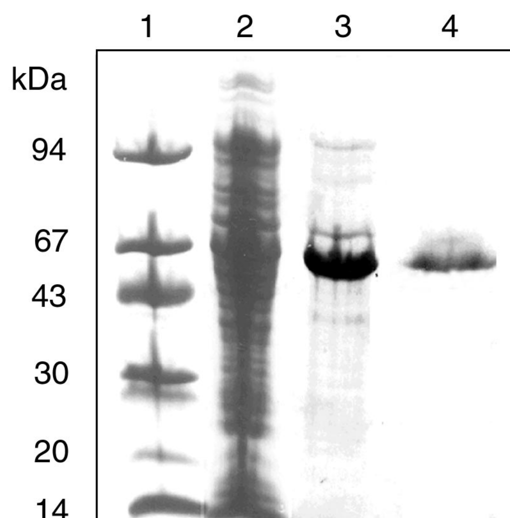

FIG. 2. Purification of the MalE-Rma fusion protein. A cell extract was prepared from a recombinant strain of *E. coli* harboring pMal-Rma and was subjected to fractionation as described in Materials and Methods. Protein samples were separated by SDS-PAGE and stained with Coomassie brilliant blue. Lane 1, polypeptide molecular size standards; lane 2, crude extract after induction; lane 3, amylose affinity column fraction; lane 4, HiTrap heparin affinity column fraction.

cleotide primers. The PCR product was purified and cloned into vector plasmid pGEM-T. When it was introduced into *E. coli* DH5α, the recombinant strain harboring the resulting plasmid, pNCTR44, displayed the MDR phenotype (Table 2), as was the case with pNCTR42. The corresponding gene was therefore designated *rma* (resistance to multiple antibiotics).

The *rma* gene and its upstream regulatory region were also amplified by PCR from the genomic DNA of wild-type *S. enterica* serovar Paratyphi B. After this region was cloned into pGEM-T, the nucleotide sequence of this region was determined and compared with that of pNCTR42, which originated from the mutant strain of *S. enterica* serovar Paratyphi B with MDR. The two nucleotide sequences were completely identical.

In vitro binding of the MalE-Rma fusion protein to a MarA operator of *E. coli*. As indicated above, the Rma protein of *S. enterica* serovar Paratyphi B exhibited a very high level of sequence identity to the RamA protein of *K. pneumoniae* and a moderate degree of identity to the MarA and SoxS proteins of *E. coli*. Furthermore, most of the residues in two helix-turn-helix structures that were shown to be important for sequence recognition and base contacts in MarA (25) are conserved in Rma of *S. enterica* serovar Paratyphi B (Fig. 1). This conservation suggested the possibility of cross interactions between these regulatory proteins and their cognate operators. To investigate this possibility, a MalE-Rma fusion protein was constructed to determine if Rma binds to MarA operator sequences. The structural gene of *rma* was amplified by PCR and fused in frame to the C terminus of the maltose binding protein (MalE) in expression vector pMAL-c2X. After induction with IPTG, the MalE-Rma fusion protein was purified from *E. coli* cells harboring the recombinant plasmid. SDS-PAGE analysis indicated that the 55-kDa MalE-Rma fusion protein (42 kDa of MalE plus 13 kDa of Rma) was purified to near homogeneity (Fig. 2).

Binding of the MalE-Rma protein to a MarA operator DNA sequence was analyzed by mobility shift assays with a labeled synthetic 26-bp double-stranded DNA fragment representing the MarA binding sequence, 5′-GGGGATTTAGCAAAACG TGGCATCGG-3′ (25). The results (Fig. 3) showed specific retardation of the DNA probe by the MalE-Rma fusion protein, which indicated that the MarA operator of *E. coli* is recognized by the Rma protein of *S. enterica* serovar Paratyphi B.

The presence of *rma* in *E. coli* causes induction of TolC synthesis and reduction of OmpF synthesis. In a previous report (8) on the characterization of the mutant strain of *S. enterica* serovar Paratyphi B with MDR, the levels of expression of several outer membrane proteins were found to be different from those of the proteins of the wild-type strain. To further investigate the effect of *rma* in *E. coli*, the outer membrane protein profiles of *E. coli* strains with and without the MDR phenotype were analyzed by SDS-PAGE. As shown in Fig. 4A, the presence of *rma*-containing plasmids (pNCTR4 and pNCTR42) in *E. coli* DH5α affects the levels of several protein bands, particularly two polypeptides with estimated molecular masses of 50 and 40 kDa. The presence of *rma* increases the level of a 50-kDa polypeptide and represses that of a 40-kDa polypeptide. The estimated molecular masses of these two polypeptides correspond quite well to those of TolC and OmpF of *E. coli*, respectively, (1). Western blot analysis with anti-TolC antibody confirmed that the 50-kDa polypeptide is TolC (Fig. 4B). Similarly, experiments with outer membranes prepared from cells grown in the presence of sodium salicylate supported the conclusion that the 40-kDa polypeptide is indeed OmpF. Exogenous salicylate has been shown specifically to suppress the synthesis of outer membrane porin OmpF (7). Such salicylate repression is evident with the 40-kDa band (Fig. 4A, lanes 2 and 5). We therefore conclude that the presence of the *S. enterica* serovar Paratyphi B *rma* gene in *E. coli* induces and represses the synthesis of TolC and OmpF, respectively.

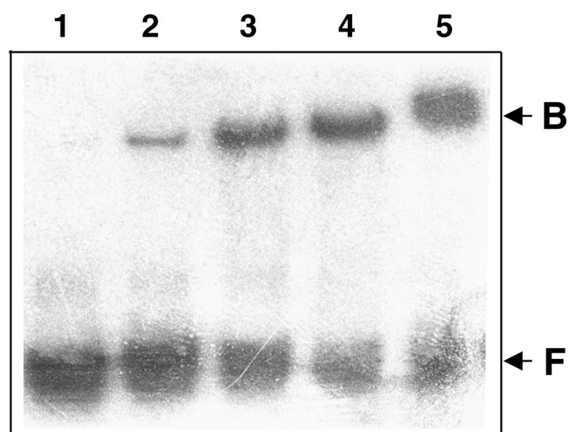

FIG. 3. Gel retardation experiments. The radioactive ^{32}P-labeled MarA operator DNA was incubated with increasing concentrations of the purified MalE-Rma fusion protein (lanes 5 to 2). Lane 1, probe control without protein. F, free DNA; B, bound DNA.

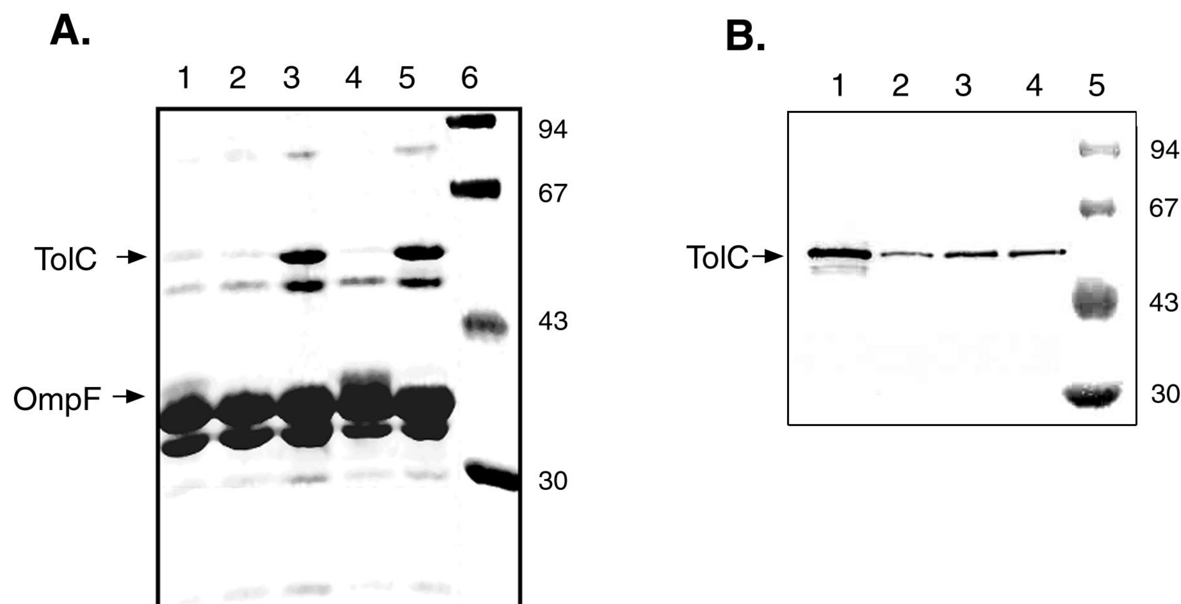

FIG. 4. Analysis of outer membrane proteins. (A) Equal amounts of outer membrane proteins were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Lane 1, *E. coli* host cells grown in LB broth; lane 2, host cells grown in LB broth in the presence of 10 mM sodium salicylate; lanes 3 to 5, cells harboring pNCTR4, pUC19, and pNCTR42, respectively; lane 6, polypeptide molecular size standards. The locations of polypeptide bands for TolC and OmpF are marked. (B) Western blot of TolC. Lane 1, cells harboring pNCTR42; lane 2, *E. coli* host cells grown in the presence of 10 mM sodium salicylate; lane 3, *E. coli* host cells; lane 4, cells harboring pUC19; lane 5, polypeptide molecular size standards. The numbers to the right of both gels are in kilodaltons.

It has been reported that salicylate also induces the *mar* operon (7). Since the *tolC* gene is activated by the *mar* system (2), one might expect TolC induction by exogenous salicylate through activation of the *mar* system. However, such induction was not observed in the present study, suggesting that induction of the *mar* system by salicylate is not at a level high enough to activate the *tolC* gene.

DISCUSSION

The results reported here indicate that overexpression of RecA confers resistance to norfloxacin but not MDR in *E. coli*. The involvement of RecA in norfloxacin resistance has been implied by the increased sensitivities of *recA* mutants to this compound (24, 33). This report shows that overexpression of RecA increases the level of resistance of *E. coli* to norfloxacin. This effect is likely mediated through the function of RecA in the SOS response. It has been suggested that norfloxacin induces a certain degree of DNA damage or interference with DNA replication (14). This, in turn, could serve as the signal to activate the coprotease activity of RecA to trigger the cascade of the SOS regulatory system (34), resulting in a higher level of resistance to norfloxacin.

In contrast, the *rma* gene of *S. enterica* serovar Paratyphi B, identified in the present study, was shown to cause an MDR phenotype in *E. coli*. The Rma protein of serovar Paratyphi B shares significant sequence identity (75 to 88%) with the RamA proteins of several other gram-negative bacteria, which have previously been reported to cause the MDR phenotype in *E. coli* (11). The unpublished sequence for *S. enterica* serovar Typhimurium shows a gene organization identical to that reported here for *S. enterica* serovar Paratyphi B (Fig. 5) with *roxA* in place of *rma*. The genes *rma* and *roxA* should be considered identical since their derived amino acid sequences are identical. By extending the comparison to other gram-negative bacteria, the gene organization in the *ramA* region of *K. pneumoniae* differs considerably from that of *rma* in *S. enterica* serovar Paratyphi B, but several factors suggest that *ramA* and *rma* are orthologues. These factors are the 88% identity of the derived polypeptide sequences, the similarities of the MDR phenotype when Rma or RamA is overexpressed in *E. coli*, and the presence of the *ybdJ* locus downstream of either gene (Fig. 5). Interestingly, no Rma counterpart can be found in *E. coli* by sequence comparison. The results of genomic comparison (Fig. 5) indicate that the gene organization surrounding *rma* of *S. enterica* serovar Paratyphi B and the corresponding region in *E. coli* are similar, with two exceptions: the absence of *rma* and the presence of *Yi81-2* of unknown function in *E. coli*.

The results of sequence comparison indicate that the Rma protein is a new member of the MarA-SoxS family of transcriptional regulators. Furthermore, the results of the mobility shift experiments reported here provide strong evidence that the Rma protein of *S. enterica* serovar Paratyphi B interacts with the MarA operator sequence. Although Rma of serovar Paratyphi B and MarA of *E. coli* share only 38% identity in their amino acid sequences, the residues of MarA that have been shown to be important for DNA interactions (25) are mostly conserved in Rma (Fig. 1). These results support the hypothesis that the Rma protein of *S. enterica* serovar Paratyphi B can substitute for MarA in affecting the expression of large number of genes in the MarA regulon and that this effect is the likely basis of its ability to cause a similar MDR phenotype in *E. coli*.

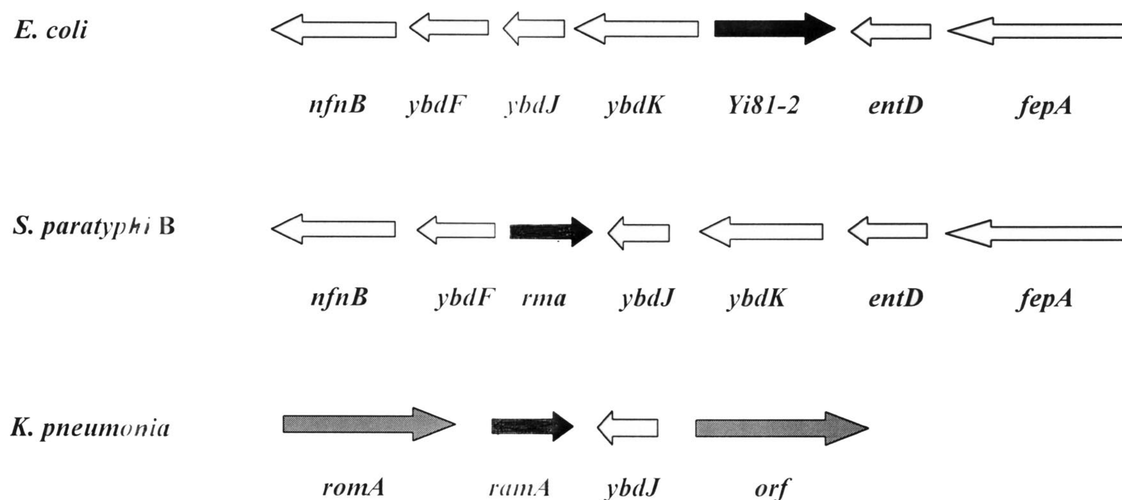

FIG. 5. Genomic comparison of the flanking regions surrounding *rma* loci. The nucleotide sequences for *E. coli*, *S. enterica* serovar Paratyphi B (*S. paratyphi* B), and *K. pneumoniae* were taken from the database at NCBI. Arrows indicate the relative sizes, locations, and transcriptional orientations of genes in the regions; and the designation for each gene is marked under the arrows.

The overexpression of TolC synthesis and the reduction in OmpF synthesis, which are associated with the *rma*-dependent MDR phenotype in *E. coli*, as reported here (Fig. 4), are similar to the changes in the outer membrane protein profile that were previously reported for the MDR phenotype conferred by the MarA proteins of other gram-negative bacteria (6, 9, 18). A recent report of a study that used microarray analysis (2) described similar changes as a result of the constitutive expression of MarA in *E. coli*. Repression of *ompF* by MarA is thought to occur indirectly through its activation of *micF*, whose RNA product interacts with *ompF* mRNA to prevent translation (4, 18). The OmpF protein is one of the major porin proteins in gram-negative bacteria (20), and TolC is required for the function of the AcrAB efflux pump, which is an essential player in the development of the MDR phenotype by MarA (9, 17, 18, 22). The similarities of the effects of *rma* of *S. enterica* serovar Paratyphi B and the *marA* genes of other gram-negative bacteria on the outer membrane protein profiles provide further support for the hypothesis that these two genes might exert their effects via similar mechanisms.

Miller and Sulavik (18) have proposed that the development of the MDR phenotype by different MarA-like regulatory proteins reflects an overlap of adaptive responses to different environmental stimuli. In general, these MarA-like proteins are small, and they are categorized as a subfamily of the AraC-XylS family (10). On the basis of the three-dimensional structure of MarA (10, 25), it is reasonable to assume that these MarA-like regulatory proteins are also monomeric in nature and have two helix-turn-helix domains for DNA binding. Most likely, they do not possess domains for oligomerization and ligand binding like other larger AraC-like regulatory proteins do. Therefore, expression of these MarA-like molecules is likely to be subject to control by other regulatory proteins in response to their cognate environmental stimulants. The MarA-MarR and SoxS-SoxR pairs represent two such examples, with *marR* and *soxR* genes located immediately upstream of the corresponding *marA* and *sox*S genes. Comparison of the genomes with the finished and unfinished sequences from NCBI showed that the *marA-marR* and the *soxS-soxR* configurations are conserved in the genome of *S. enterica* serovar Paratyphi B. However, analysis of the gene organization in the regions flanking the *rma* locus of *S. enterica* serovar Paratyphi B revealed no candidate for a cognate regulator of Rma (Fig. 5).

Although overexpression of Rma causes MDR in *E. coli*, the nucleotide sequences of the *rma* structural gene and its putative regulatory region are identical between *S. enterica* serovar Paratyphi B mutant M95 with MDR and its parent strain. These results suggest that it is overexpression of the *rma* gene that results in the MDR phenotype and raise the possibility that the phenotype of M95 is the result of a mutation that causes the induction of *rma* on the chromosome. Such a mutation could be in a cognate regulatory protein that is yet to be identified.

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