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Salmonella Gene *rma* (*ramA*) and Multiple-Drug-Resistant *Salmonella enterica* Serovar Typhimurium

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MarA and its homologue, RamA, have been implicated in multidrug resistance (MDR). *RamA* overexpression in *Salmonella enterica* serovar Typhimurium and *Escherichia coli* conferred MDR independently of *marA*. Inactivation of *ramA* did not affect the antibiotic susceptibilities of wild-type *S. enterica* serovar Typhimurium or 15 unrelated clinical MDR isolates. Thus, *ramA* overexpression is not a common MDR mechanism in *Salmonella*.

Multiple antibiotic resistance in *Salmonella enterica* serovar Typhimurium, an etiologic agent of food-borne enterocolitis in humans, is becoming a serious health problem. A multiple-drug-resistant (MDR) phenotype can likely develop in gram-negative microorganisms by many mechanisms (4); most of these have been elucidated in *Escherichia coli*. Among other mechanisms, an important route involves activation of the *mar* locus: MarA, the transcriptional activator of this locus, mediates drug resistance by causing decreased expression of the porin OmpF and overexpression of the multidrug efflux pump ArcB (1, 9). Additional genetic mechanisms of MDR have been proposed. For instance, homologues of MarA, such as Rob and SoxS, have been shown to bind to the *mar* box; and constitutive *soxS* or *rob* mutants display MDR as well (3, 8). George and coworkers (2) identified the *ramA* gene in MDR *Klebsiella pneumoniae* and suggested that the MDR phenotype of this strain was caused by constitutive overexpression of RamA. Because RamA displays close homology to MarA, SoxS, and Rob, the suggestion was made that RamA mediates MDR in *Klebsiella* via activation of the *mar* locus. Recently, a gene identical to *ramA* was also identified in *S. enterica* serovar Paratyphi B and was designated *rma* (11). In this report, we describe a gene identical to *ramA* (*rma*) in *S. enterica* serovar Typhimurium that, when overexpressed on a plasmid in *E. coli* which lacks *ramA*, conferred an MDR phenotype to this bacterium and investigate whether this gene has a role in MDR in *S. enterica* serovar Typhimurium.

The strains and plasmids used in this study are listed in Table 1. *E. coli marA* mutants were kindly provided by S. L. Levy (6). The MDR *S. enterica* serovar Typhimurium strains were obtained from the surveillance collection of CIDC-Lelystad, Lelystad, The Netherlands, and are representatives of unrelated clinical MDR isolates obtained in The Netherlands over a 2-year period. The *ramA* gene was inactivated in these strains by transduction with a P22 lysate of the *ramA*::kanamycin *Salmonella* mutant (10).

To induce expression of RamA, the RamA-coding sequence was ligated into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible vector pTrcHisA (Invitrogen) by standard techniques. For constitutive overexpression, *ramA* was ligated into pBluescript (Stratagene).

Disk diffusion assays were performed as follows. End-log-phase bacteria (optical density at 600 nm, 0.8) were diluted 1:10 in phosphate-buffered saline and plated on minimal M9 medium. For *E. coli* the plates were supplemented with thiamine (0.01%) and Casamino Acids (0.1%). If required, ampicillin (50 μg/ml) or IPTG (0.1 mM) was added. Cotton disks containing antibiotics were placed in the centers of the plates. After overnight incubation at 37°C, the bacterium-free zone was determined as a measure of resistance. The disk diffusion assay was used to test the antibiotic susceptibilities of the bacterial mutant strains, for which the classical MIC broth microdilution method is not adequate (5).

The MICs for the clinical *Salmonella* isolates were deter-

TABLE 1. *Salmonella* strains and plasmids used in this study

Strain or plasmid	Characteristic	Origin or reference
S. enterica serovar Typhimurium		
14028s	Wild type	ATCC[a]
14028Δ*ramA*	*ramA* null mutant	10
E. coli		
MC1061	Wild type	ATCC
AG100	Wild type	6
AG100kana	Δ*marA*	6
Plasmids		
pBluescript	Cloning vector	Stratagene
pB1-*ramA*	Constitutive RamA expression	10
pTrcHisA	IPTG-inducible plasmid	Invitrogen
pTrcHisA-*ramA*	Inducible RamA expression	10

[a] ATCC, American Type Culture Collection.

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TABLE 2. Antibiotic susceptibilities of *E. coli* and *S. enterica* serovar Typhimurium strains

Strain	Zone of inhibition (mm)[a]				
	NAL	CIP	CHL	TET	GEN
S. enterica serovar Typhimurium					
14028s	40 ± 1	48 ± 2	41 ± 1	42 ± 2	ND[d]
14028s Δ*ramA*	38 ± 1	47 ± 2	40 ± 1	42 ± 2	ND
14028s + pTrcHisA-*ramA*[b]					
No IPTG	21 ± 1	40 ± 2	29 ± 1	37 ± 1	ND
IPTG added	15 ± 1	33 ± 1	12 ± 2	30 ± 2	ND
E. coli					
MC1061 + pB1[c]	ND	42 ± 2	30 ± 2	ND	ND
MC1061 + pB1-*ramA*	ND	32 ± 1	24 ± 2	ND	ND
AG100	23 ± 1	39 ± 1	35 ± 1	31 ± 1	30 ± 1
AG100kana (*marA*::*kan*)	23 ± 1	41 ± 2	35 ± 1	30 ± 1	30 ± 1
AG100 + pHisA-*ramA*					
No IPTG	21 ± 1	39 ± 1	34 ± 1	30 ± 1	30 ± 1
IPTG added	15 ± 1	36 ± 1	21 ± 2	27 ± 1	30 ± 1
AG100kana (*marA*::*kan*) + pHisA-*ramA*					
No IPTG	23 ± 1	40 ± 1	34 ± 2	30 ± 1	30 ± 1
IPTG added	17 ± 1	33 ± 1	22 ± 1	25 ± 1	30 ± 1

[a] Zones of growth inhibition around cotton disks (diameters, 6 mm) were determined by the disk diffusion assay on standardized M9 minimal medium agar plates; the following dosages were added to the disks: nalidixic acid (NAL), 130 μg; ciprofloxacin (CIP) 10 μg; chloramphenicol (CHL), 300 μg; tetracycline (TET), 80 μg; gentamicin (GEN), 100 μg. The (CIP), mean and standard deviations of four independent experiments are given.
[b] *ramA* gene expressed on IPTG-inducible plasmid pTrcHisA (Invitrogen).
[c] pB1, plasmid pBluescript.
[d] ND, not done.

mined by the broth microdilution method, according to the NCCLS guidelines (7). An E-test was performed by standard procedures for determination of tetracycline resistance.

Overexpression of RamA confers MDR in *S. enterica* serovar Typhimurium. Given the homology between RamA and MarA and the findings for *Klebsiella* and *S. enterica* serovar Paratyphi B, we investigated the ability of RamA to confer resistance to various unrelated antibiotics in *S. enterica* serovar Typhimurium by means of disk diffusion assays. We induced expression of RamA in wild-type *Salmonella* with the IPTG-inducible *ramA* plasmid and expressed RamA in *E. coli* with pBl-*ramA*. Both microorganisms displayed an MDR phenotype after overexpression of RamA (Table 2), which is in accordance with the published results of George et al. (2) on the expression of RamA in *E. coli*. Of note, the latter bacterium lacks *ramA*, and we found that *ramA* is highly confined to *S. enterica* serovars (10) and is not present in the genomes of many other members of the family *Enterobacteriaceae*, with the notable exceptions of *K. pneumoniae* and *Enterobacter cloacae*.

The MDR phenotype mediated by RamA is independent of MarA. Yassien et al. (11) showed that RamA (Rma) of *S. enterica* serovar Paratyphi B is a DNA binding protein that binds to the *mar* box. MarA is a transcriptional activator for *marRAB* and binds to the *mar* box located within *marO*. Homologues of MarA, such as SoxS, Rob, and RamA, have been shown to bind to the *mar* box and also to upregulate expression of the *mar* locus (3,8,11). Thus, on the basis of experiments with *E. coli*, Yassien et al. (11) hypothesized that RamA can substitute for MarA and directly activate MarA-controlled genes, leading to an MDR phenotype. An alternative explanation for their data would be that the MDR phenotype conferred by overexpression of RamA is MarA dependent. To investigate this issue we expressed RamA on an IPTG-inducible multicopy plasmid in a *marA*-negative *E. coli* mutant and its parental strain. As assessed by disk diffusion assays, in both wild-type *E. coli* and the *marA*-negative mutant, RamA significantly ($P < 0.025$) increased the levels of resistance to multiple unrelated antibiotics and conferred an MDR phenotype (Table 2). This result demonstrates that in *E. coli* RamA can mediate an MDR phenotype independently of a functionally intact *marA*, likely by direct activation of MarA-controlled genes.

The antibiotic susceptibility of wild-type *Salmonella* is not affected by inactivation of *ramA*. Next, we assayed the resistance of *ramA* null mutants of *S. enterica* serovar Typhimurium to multiple unrelated antibiotics. These strains were obtained by gene replacement with suicide vector pGP704, which contains *ramA* inactivated by a kanamycin cassette (10). Compared with the wild-type parental *Salmonella* strain, the null mutants did not display increased susceptibilities to tetracycline, chloramphenicol, ciprofloxacin, or nalidixic acid (Table 2). The identical susceptibilities of the *Salmonella* strains to, for instance, ciprofloxacin were confirmed by E-test on Iso-Sensitest agar plates, with the MICs for all strains being 0.032 to 0.064 mg/liter.

The MDR phenotype of clinical isolates of *Salmonella* is not affected by inactivation of *ramA*. Further evidence that a functionally intact *ramA* is dispensable for the expression of an MDR phenotype was obtained in experiments with 15 clinical *S. enterica* serovar Typhimurium isolates (including strain 12 DT104), all of which displayed an MDR phenotype, as defined by resistance to at least three unrelated antibiotics. These strains were obtained from the Dutch national surveillance collection of CIDC-Lelystad and are representative of unrelated clinical MDR isolates obtained in The Netherlands over a 2-year period. In these strains the *ramA* gene was inactivated by transduction with a P22 lysate of the *ramA*::kanamycin *Salmonella* mutant. The MDR phenotype was not reversed to a non-MDR, susceptible phenotype in any of these strains (Table 3), as determined by assays for MICs. In more than 270 assays for MICs, only 2 indicated a change in the MIC of more than 2 dilution steps by the broth microdilution method, according to the NCCLS guidelines (7). The MICs of doxycycline, tetracycline, and florfenicol for six MDR strains showed slight decreases; however, according to the NCCLS guidelines, the interpretation of the final MICs still indicated a resistant phenotype.

In conclusion, overexpression of RamA in *E. coli* and *S. enterica* serovar Typhimurium confers an MDR phenotype in a MarA-independent manner that is likely mediated by direct activation of *mar*-regulated genes, although formal proof for this is not yet available. However, inactivation of *ramA* does not lead to enhanced antibiotic susceptibility and does not reverse the antibiotic resistance phenotypes of 15 unrelated clinical MDR *S. enterica* serovar Typhimurium isolates. Thus,

TABLE 3. MICs for MDR *S. enterica* serovar Typhimurium strains and their *ramA* knock-out mutants

Salmonella strain	MIC (mg/liter)								
	Amoxicillin	Gentamicin	Doxycycline	Trimethoprim	Tetracycline	Flumequine	Ciprofloxacin	Florfenicol	Chloramphenicol
14028s	1	1	2	≤0.5	1	≤0.5	<0.06	4	8
14028s Δ*ramA*	1	1	2	≤0.5	1	≤0.5	<0.06	4	8
MDR strains									
1	>32	≤0.25	16	≤0.5	32	≤0.5	<0.03	128	>128
1 Δ*ramA*	>32	0.5	16	≤0.5	24	≤0.5	<0.06	64	>128
2	>32	≤0.25	16	>64	>256	1	<0.06	128	>128
2 Δ*ramA*	>32	0.5	8	>64	48	≤0.5	<0.06	32	>128
3	>32	0.5	16	≤0.5	96	≤0.5	<0.03	128	>128
3 Δ*ramA*	>32	0.5	4	≤0.5	24	≤0.5	<0.06	64	>128
4	>32	≤0.25	16	≤0.5	32	1	<0.03	32	>128
4 Δ*ramA*	>32	1	8	≤0.5	24	≤0.5	<0.06	32	>128
5	>32	≤0.25	16	1	>256	2	<0.06	64	>128
5 Δ*ramA*	>32	≤0.25	8	≤0.5	24	≤0.5	<0.06	16	>128
6	>32	0.5	16	≤0.5	32	1	<0.03	32	>128
6 Δ*ramA*	>32	≤0.25	4	≤0.5	32	≤0.5	<0.06	32	>128
7	>32	≤0.25	16	≤0.5	48	≤0.5	<0.03	32	>128
7 Δ*ramA*	>32	0.5	4	≤0.5	16	≤0.5	<0.06	16	>128
8	>32	0.5	16	≤0.5	48	≤0.5	<0.03	32	>128
8 Δ*ramA*	>32	0.5	8	≤0.5	24	≤0.5	<0.06	16	>128
9	>32	0.5	16	≤0.5	96	≤0.5	<0.03	32	>128
9 Δ*ramA*	>32	0.5	4	≤0.5	24	≤0.5	<0.06	32	>128
10	>32	0.5	64	>64	256	≤0.5	<0.03	2	>128
10 *ramA*	>32	0.5	32	>64	256	1	<0.06	4	>128
11	>32	≤0.25	32	≤0.5	>256	64	>4	4	>128
11 *ramA*	>32	0.5	32	≤0.5	256	32	8	4	>128
12	>32	≤0.25	16	≤0.5	48	≤0.5	<0.03	64	>128
12 *ramA*	>32	0.5	8	≤0.5	32	≤0.5	<0.06	16	>128
13	>32	0.5	32	>64	256	≤0.5	<0.03	4	>128
13 *ramA*	>32	0.5	32	>64	256	≤0.5	<0.06	4	>128
14	>32	≤0.25	16	≤0.5	96	1	<0.03	64	>128
14 *ramA*	>32	0.5	16	≤0.5	128	≤0.5	<0.06	32	>128
15	>32	≤0.25	16	≤0.5	32	≤0.5	<0.03	64	>128
15 *ramA*	>32	0.5	8	≤0.5	24	1	<0.06	32	>128

[a] According to NCCLS guideline M2-A7, the MIC breakpoints are as follows: for amoxicillin (the criteria for ampicillin were used), sensitive, ≤8 mg/liter; resistant, ≥32 mg/liter; for gentamicin, sensitive, ≤4 mg/liter; resistant, ≥8 mg/liter; for doxycycline, sensitive, ≤4 mg/liter; resistant, ≥16 mg/liter; for flumequine (the criteria for oxolinic acid were used), sensitive, ≤4 mg/liter; resistant, ≥8 mg/liter; for tetracycline, sensitive, ≤4 mg/liter; resistant, ≥16 mg/liter (values for tetracycline were determined by E-test); for ciprofloxacin, sensitive, ≤2 mg/liter; resistant, ≥4 mg/liter; for chloramphenicol, sensitive, ≤64 mg/liter; resistant, ≥128 mg/liter. According to NCCLS guideline M31-A2, MIC breakpoints for florfenicol are as follows: sensitive, ≤2 mg/liter; resistant, ≥8 mg/liter.

the findings for *Salmonella* rule against a common role of this gene in the MDR phenotypes of clinical *Salmonella* isolates.

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