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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>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristic</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. enterica serovar Typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14028s</td>
<td>Wild type</td>
<td>ATCC</td>
</tr>
<tr>
<td>14028ΔramA</td>
<td>ramA null mutant</td>
<td>10</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC1061</td>
<td>Wild type</td>
<td>ATCC</td>
</tr>
<tr>
<td>AG100</td>
<td>Wild type</td>
<td>6</td>
</tr>
<tr>
<td>AG100kana</td>
<td>ΔmarA</td>
<td>6</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript</td>
<td>Cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBl-ramA</td>
<td>Constitutive RamA expression</td>
<td>10</td>
</tr>
<tr>
<td>pTrcHisA</td>
<td>IPTG-inducible plasmid</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pTrcHisA-rmaA</td>
<td>Inducible RamA expression</td>
<td>10</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Zone of inhibition (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAL</td>
</tr>
<tr>
<td><em>S. enterica</em> serovar Typhimurium</td>
<td></td>
</tr>
<tr>
<td>14028s</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>14028s ΔramA</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>14028s + pTrcHisA-ramA</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>No IPTG</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>IPTG added</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>AG100</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>AG100kana (marA::kan)</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>AG100 + pHisA-ramA</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>No IPTG</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>IPTG added</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>AG100kana (marA::kan) +</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>pHisA-ramA</td>
<td>17 ± 1</td>
</tr>
</tbody>
</table>

* 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. | pB1, plasmid pBluescript. *(a) marA gene expressed on IPTG-inducible plasmid pTrcHisA (Invitrogen).* | 

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 Paratyphii 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. Yassin et al. (11) showed that RamA (Rma) of *S. enterica* serovar Paratyphii 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*, Yassin 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 used RamA on an IPTG-inducible multicity 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 *E. coli* 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-Sensitase 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 CDC-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,
the findings for Salmonella rule against a common role of this gene in the MDR phenotypes of clinical Salmonella isolates.

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