MAJOR ARTICLE

Effects of Natural Mutations in the *ramRA* Locus on Invasiveness of Epidemic Fluoroquinolone-Resistant *Salmonella enterica* Serovar Typhimurium Isolates

Etienne Giraud,[1,2] Sylvie Baucheron,[1,2] Isabelle Virlogeux-Payant,[1,2] Kunihiko Nishino,[3] and Axel Cloeckaert[1,2]

[1]INRA, UMR1282 Infectiologie et Santé Publique, Nouzilly, and [2]Université François Rabelais de Tours, UMR1282 Infectiologie et Santé Publique, Tours, France; and [3]Laboratory of Microbiology and Infectious Diseases, Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, Japan

Background. Fluoroquinolone (FQ) resistance is increasing worldwide among *Salmonella* species. Among the mechanisms involved, increased efflux via the tripartite AcrAB-TolC efflux system is mainly modulated through control of expression via the *ramRA* regulatory locus gene products. Interestingly, in some reference strains these have also been experimentally shown to regulate cell invasion–related genes of the type III secretion system 1 (T3SS-1). In this study, we investigated whether natural mutations occurring in this locus in FQ-resistant *S. enterica* serovar Typhimurium epidemic clones resulted in the same effects.

Methods. Quantitative reverse transcription polymerase chain reaction and cell invasion assays were used to study 3 clinical FQ-resistant *S.* Typhimurium isolates representative of the DT104 and DT204 epidemic clones. For comparison, 3 control reference quinolone-susceptible strains were included.

Results. As previously shown, the investigated mutations altering RamR or its DNA-binding site increased expression of efflux genes dependently on *ramA*. However, the decreased expression of T3SS-1 genes previously reported was not always observed and seemed to be dependent on the genetic background of the FQ-resistant isolate. Indeed, a *ramA*-dependent decreased invasion of intestinal epithelial cells was only observed for a particular clinical *ramR* mutant.

Conclusions. *ramRA* mutations occurring in clinical FQ-resistant *S.* Typhimurium isolates may negatively modulate their invasiveness but this is strain-dependent.

Keywords. *Salmonella*; *ramA*; *ramR*; cell invasion; multidrug resistance; efflux; fluoroquinolone; type III secretion-system 1; transcriptional regulation.

Salmonella is frequently involved, worldwide, in foodborne human diseases. The antimicrobials of choice to treat severe salmonellosis are fluoroquinolones (FQs) and extended-spectrum cephalosporins, especially since epidemic clones such as *Salmonella enterica* serotype Typhimurium DT104, which emerged and spread during the 1990s, showed acquired resistance to different families of antimicrobial agents [1, 2]. Up to now, clinical FQ resistance remains less frequent in *Salmonella* than in other enterobacteria, possibly because of a fitness burden that may limit the emergence and spread of highly resistant clones [3, 4]. However, FQ resistance is increasingly observed among particular *Salmonella* clones of various serotypes, such as the recent *Salmonella enterica* serotype Kentucky ST198 clone, and is thus becoming a public health threat [5–10].

Mechanisms responsible, when combined in a single *Salmonella* strain, for high-level FQ resistance include point mutations in the genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC*

Received 6 June 2012; accepted 11 October 2012; electronically published 10 December 2012.

Presented in part: 3rd ASM Conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens, poster 75B, Aix-en-Provence, France, 26–29 June 2012.

Correspondence: Etienne Giraud, PhD, INRA, UMR1282 Infectiologie et Santé Publique, F-37380 Nouzilly, France (etienne.giraud@tours.inra.fr).

The Journal of Infectious Diseases 2013;207:794–802

© The Author 2012. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com.

DOI: 10.1093/infdis/jis755

Downloaded from https://academic.oup.com/jid/article/207/5/794/1079166 by guest on 23 April 2024

and *parE*), plasmid-mediated protection of these target enzymes, and enhanced active efflux due to the overproduction of the broad-spectrum AcrAB-TolC efflux system [1]. The plasmid-mediated quinolone-resistance determinant *aac(6')-Ib-cr*, which encodes an FQ-modifying enzyme, was also reported in *Salmonella* isolates [11]. Enhanced active efflux was proposed as the primary mechanism (ie, as the first to occur) in the development of ciprofloxacin resistance in *S.* Typhimurium [12]. Indeed, AcrAB-TolC overproduction and consequent decreased susceptibility to multiple antibiotics is frequently observed in FQ-resistant strains. It was shown in *S.* Typhimurium that *ramRA* is the main regulatory locus of AcrAB-TolC. Activation of the *acrAB* operon and *tolC* gene transcription is achieved through the direct binding of RamA, a positive regulator of the AraC/XylS family, to the operator regions of these genes [13, 14]. In susceptible strains, the transcription of *ramA* is repressed by RamR, which is encoded by the *ramR* gene located immediately upstream of *ramA* and divergently transcribed from this latter [15]. RamA is determinant in the acquisition of a phenotype of decreased susceptibility to multiple antibiotics, as evidenced by the inability to select spontaneous mutants with such phenotype from a *ramA*-inactivated strain after exposure to ciprofloxacin [16]. In contrast, RamA overproduction associated with mutations that alter the RamR repressor or its binding to the *ramA* promoter was shown to mediate a 2–4-fold increase of resistance levels in clinical and experimentally selected strains of *S.* Typhimurium and other *S. enterica* serovars [17–23].

Besides efflux-related genes, the *ramA* regulon comprises a large set of genes with diverse functions, including virulence-related genes [24, 25]. In particular, Bailey et al showed that the overexpression of *ramA* leads to a decreased expression of genes of the *Salmonella* pathogenicity island 1 (SPI-1), which is involved in the biogenesis of the type III secretion system 1 (T3SS-1) required for host cell invasion [24, 26]. A matter of concern is that *ramRA* mutations resulting in enhanced efflux-mediated resistance to multiple antibiotics, including FQ, may also influence the virulence of clinical strains. Here, we addressed the effect on the transcription of efflux-related (*acrAB* and *tolC*) and invasion-related (*hilA*, *invA*, and *sipA*) genes and on the invasiveness of natural *ram* mutations involved in the decreased susceptibility to multiple antibiotics among 3 FQ-resistant *S.* Typhimurium clinical isolates of the DT104 and DT204 epidemic clones. For comparison, experimental deletion mutants of the 14028s (DT133) reference strain and of 2 quinolone-susceptible DT104 isolates were also studied.

METHODS

Bacterial Strains

All the strains used in this study are listed in Table 1. The *S.* Typhimurium quinolone-resistant multidrug-resistant (MDR) isolates 102SA00, BN10055, and 543SA98 and the susceptible strain S/921495, as well as their respective derivatives, were previously described [17]. Additional strains were the wild-type strain ATCC14028s and its Δ*ramR* and Δ*ramA::kan* mutants, as well as the previously described quinolone-susceptible MDR 1948SA96 isolate and its Δ*ramR*, Δ*ramA::kan* and Δ*ramRA::kan* mutants [27]. These deletion mutants were constructed by the Datsenko and Wanner gene inactivation method, using previously described primers [17, 28]. All the strains were routinely cultivated at 37°C in Luria-Bertani (LB) medium, supplemented with kanamycin at 50 µg/mL when necessary. In these culture conditions, no differences in the growth rates were observed between the parental strains and their derivatives.

Minimum Inhibitory Concentration (MIC) Determinations

The MICs of nalidixic acid (Nal), flumequine (Flu), enrofloxacin (Enr), ciprofloxacin (Cip), chloramphenicol (Cm), florfenicol (Ff), and tetracycline (Tc) were determined by the standard agar doubling dilution method, as described previously and according to the guidelines of the CASFM (available at: http://www.sfm-microbiologie.org/UserFiles/file/CASFM/casfm_2010.pdf).

Gene Expression Analysis by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Bacteria were grown until the mid-log phase (optical density at 600 nm [OD_{600}], 0.6) and harvested by centrifugation. Pelleted cultures were stabilized with RNAprotect Bacteria Reagent (Qiagen) and stored at −80°C until use. Total RNA were extracted using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Removal of residual genomic DNA was performed using the Turbo DNA-free kit (Ambion) and checked by negative PCR amplification of a chromosomal sequence. RNA integrity was examined by electrophoresis on 1% agarose gel. Total RNA (1.5 µg) was reverse transcribed using random hexamers and the Superscript III First Strand Synthesis System (Applied Biosystems). The expression level of each gene of interest was calculated as the average of 3 independent complementary DNA (cDNA) samples. For each cDNA sample and each gene, qRT-PCR runs were performed in duplicated wells. Primers used for qRT-PCR are listed in Table 2. The cycling conditions were as follows: 95°C for 5 minutes followed by 40 cycles of 95°C for 10 seconds and 60°C for 15 seconds. After each run, amplification specificity and the absence of primer dimers were examined using a dissociation curve acquired by heating the PCR products from 60°C–95°C. The relative quantities of transcripts were determined using the standard curve method and normalized against the geometric mean of 3 reference genes (*gmk*, *gyrB*, and *rrs*). A 2-tailed Student *t* test was used to assess significance, using a P value of $< .05$ as a cutoff.

Downloaded from https://academic.oup.com/jid/article/207/5/794/1079166 by guest on 23 April 2024

Table 1. *Salmonella enterica* Serovar Typhimurium Strains Used in This Study

Strain	Phage Type	Antimicrobial MIC, µg/mL							QRDR Substitution(s)			Natural Mutations at *ramRA* Locus	Source
		Nal	Flu	Enr	Cip	Cm	Ff	Tc	GyrA	GyrB	ParC		
Quinolone-resistant isolates and derivatives													
102SA00	DT204	>4096	2048	64	32	512	16	256	S83A; D87N	S464F	S80I	IS*1* in *ramR*	[17]
102SA00pRamR	DT204	4096	512	16	8	256	4	256	S83A; D87N	S464F	S80I	IS*1* in *ramR*	[17]
102SA00Δ*ramA*::*kan*	DT204	4096	512	16	8	256	4	256	S83A; D87N	S464F	S80I	IS*1* in *ramR*	[17]
BN10055	DT104	>4096	64	2	1	1024	128	256	S83Y	…	…	Altered RamR binding site	[17]
BN10055pRamR	DT104	>4096	64	2	1	1024	128	256	S83Y	…	…	Altered RamR binding site	[17]
BN10055Δ*ramA*::*kan*	DT104	512	16	0.5	0.25	128	32	64	S83Y	…	…	Altered RamR binding site	[17]
543SA98	DT104	1024	32	2	0.5	512	512	128	S83F	…	…	Frameshift in *ramR*	[17]
543SA98pRamR	DT104	512	8	0.5	0.125	128	128	32	S83F	…	…	Frameshift in *ramR*	[17]
543SA98Δ*ramA*::*kan*	DT104	512	8	0.5	0.125	128	128	32	S83F	…	…	Frameshift in *ramR*	[17]
Quinolone-susceptible strains and derivatives													
14028s	DT133	4	0.5	0.06	0.03	4	4	0.5	…	…	…	…	ATCC
14028sΔ*ramR*	DT133	8	2	0.125	0.06	8	16	2	…	…	…	…	[15]
14028sΔ*ramA*::*kan*	DT133	4	0.5	0.03	0.015	2	2	0.25	…	…	…	…	This study
S/921495	DT104	4	0.5	0.030	0.015	4	4	1	…	…	…	…	[17]
S/921495Δ*ramR*	DT104	16	2	0.125	0.060	16	16	4	…	…	…	…	[17]
1948SA96	DT104	4	0.5	0.03	0.015	256	32	32	…	…	…	…	[33]
1948SA96Δ*ramR*	DT104	8	2	0.06	0.03	>256	64	128	…	…	…	…	This study
1948SA96Δ*ramA*::*kan*	DT104	4	0.5	0.015	0.0075	128	32	16	…	…	…	…	This study
1948SA96Δ*ramRA*::*kan*	DT104	4	0.5	0.015	0.0075	128	32	16	…	…	…	…	This study

High-level resistance in isolates BN10055 and 543SA98 to phenicols and tetracycline is due to the presence of the *floR* and *tet*(G) genes on *Salmonella* genomic island 1 (SGI1). High-level resistance of isolate 102SA00 to chloramphenicol and tetracycline is due to the presence of specific resistance genes on a multidrug-resistance plasmid.

Downloaded from https://academic.oup.com/jid/article/207/5/794/1079166 by guest on 23 April 2024

Table 2. Quantitative Reverse Transcription Polymerase Chain Reaction Primers Used in This Study

Primer	Oligonucleotide Sequence (5′ to 3′)
gmk-f	TTGGCAGGGAGGCGTTT
gmk-r	GCGCGAAGTGCCGTAGTAAT
gyrB-f	TCTCCTCACAGACCAAAGATAAGCT
gyrB-r	CGCTCAGCAGTTCGTTCATC
rrs-f	CCAGCAGCCGCGGTAAT
rrs-r	TTTACGCCCAGTAATTCCGATT
ramA-f	GCGTGAACGGAAGCTAAAAC
ramA-r	GGCCATGCTTTTCTTTACGA
acrA-f	GAAACCGCACGTATCAACCT
acrA-r	CCTGTTTCAGCGAACCATTT
tolC-f	GCCCGTGCGCAATATGAT
tolC-r	CCGCGTTATCCAGGTTGTTG
hilA-f	CATGGCTGGTCAGTTGGAG
hilA-r	CGTAATTCATCGCCTAAACG
invA-f	GGCGCCAAGAGAAAAAGATG
invA-r	CAAATATAACGCGCCATTGCT
sipA-f	TTTGGCTGTACGTTAGATCCGTTA
sipA-r	CCGCCGCTTTGTCAACA

Protein Extractions and Western Blots

Secreted or intracellular proteins were recovered as previously described from bacterial cultures in LB broth or LB broth containing 0.3 M NaCl, until the OD_{600} reached 0.6 and 1.8–2.0, respectively [29]. To ensure similar precipitation and loading of the secreted proteins, β-lactoglobulin was added to each culture supernatant. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and either revealed by colloidal Coomassie brilliant blue G-250 staining or transferred onto a nitrocellulose membrane (Protran; Schleicher and Schuell). Membranes were probed either with a polyclonal rabbit anti-SipA antiserum (1:2000 [30]) or a polyclonal mouse anti-Hsp60 antiserum (1:6000, Assaydesigns-Stressgen). Hsp60 is a cytoplasmic protein that is constitutively expressed by bacteria and thus served as a loading control in the experiments. Signals were revealed using a peroxidase-goat anti rabbit serum (1:10 000, Dako) or a peroxidase-rabbit anti mouse serum (1:5000, Dako) and the SuperSignal West Dura Extended Duration Substrate as described by the manufacturer (Thermo Scientific).

Invasion and Adhesion Assays

Invasions assays were performed on Caco-2 intestinal epithelial cells as previously described [31]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated fetal bovine serum, 1% nonessential amino acids, and 1% antibiotic solution (Gibco, Invitrogen). They were seeded at 2×10^5 cells/well in a 24-well plate (Falcon) and grown to confluence at 37°C under 5% CO_2 in the medium described above. Antibiotic was removed 24 hours before performing the invasion assays. After growth to an OD_{600} of 0.6 in LB broth, bacteria were inoculated on Caco-2 monolayers at a multiplicity of infection of 30, and the plates were incubated for 30 minutes. The bacteria-containing medium was then removed from the wells, and the monolayers were washed with phosphate-buffered saline (PBS). Cells were incubated for 1.5 hours with DMEM supplemented with 100 µg/mL gentamicin. Cells were washed with PBS and lysed with sterile ultrapure water for 30 minutes. Serial dilutions of cell lysates were plated on LB agar. The percentage of penetrating bacteria was calculated on the basis of the ratio of the counted colon-forming units to the bacterial inoculum. Three replicates were performed for each bacterial strain.

In experiments conducted with epithelial HT-29 cells, adhesion assays were performed in addition to invasion assays. A multiplicity of infection of 10 was used, and bacteria were maintained in contact with cells for 1.5 hours. To measure adhesion, cells were washed and lysed before gentamicin treatment. Viable intracellular and extracellular bacteria were counted after plating serial dilutions on LB agar. Two independent assays were performed in duplicate. Data were compared using 1-way analysis of variance and then analyzed by the Tukey-Kramer multiple comparisons test, using Systat 13 software.

RESULTS

Effect of *ramRA* Mutations on Expression of Efflux Genes and Resistance Levels

We tested the influence of natural and experimental *ramRA* mutations on the expression of efflux-related genes (*ramA*, *acrA*, and *tolC*) and on the resistance to quinolones and other antimicrobials substrates of the AcrAB-TolC multidrug efflux system. For that purpose, we used 3 FQ-resistant *S.* Typhimurium clinical isolates of phage types DT104 or DT204 with natural *ramRA* mutations, as well as 3 experimental deletion mutants of *ramR*, *ramA*, or the whole *ramRA* locus.

We previously reported that the complementation with a functional *ramR* gene of the 2 FQ-resistant isolates with natural mutations in *ramR* (DT204 strain 102SA00 and DT104 strain 543SA98) resulted in a 2- or 4-fold decrease in antimicrobial MICs (Table 1) [17]. As shown by qRT-PCR, these MIC decreases correlated with decreases in the transcript levels of *ramA* (by 11- and 8-fold, respectively) and of the *acrA* (by 1.4- and 2.0-fold) and *tolC* (by 1.5- and 2.3-fold) efflux genes (Figure 1*A*). For the FQ-resistant isolate BN10055, a complementation effect was observed neither at the phenotypic level nor at the level of efflux genes expression. This was expected because this isolate bears a 2-bp deletion at the RamR binding site located in the *ramA* operator region but no mutation in *ramR* itself. In contrast, the inactivation of *ramA* abolished the effect of mutations in *ramR* or in the

Downloaded from https://academic.oup.com/jid/article/207/5/794/1079166 by guest on 23 April 2024

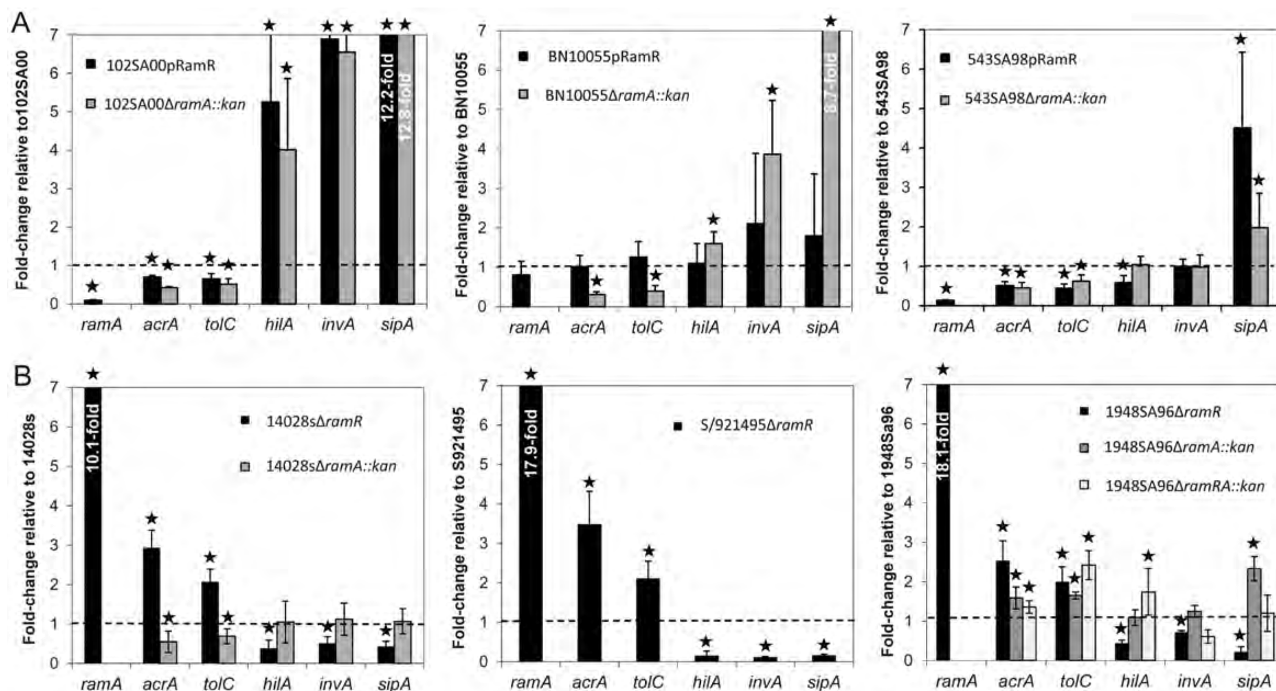

Figure 1. Quantitative reverse transcription polymerase chain reaction analysis of the transcript levels of efflux- or invasion-related genes in fluoroquinolone (FQ)–resistant isolates harboring natural *ramRA* mutations and their derivatives (*A*) and in experimental *ramR*, *ramA*, and *ramRA* deletion mutants of quinolone-susceptible strains (*B*). Bars indicate the messenger RNA fold-changes observed in derivatives compared to their parental strain. The dashed lines indicate a fold-change of 1 (ie, no change). Error bars represent SDs. Stars indicate significant differences ($P < .05$).

RamR binding site. Indeed, the deletion of *ramA* isolates led, in the 3 clinical isolates, to a decrease of the transcripts levels of *acrA* (by 2.2–3.2-fold) and *tolC* (by 1.6–2.5-fold). This is consistent with the 2- or 4- fold decrease in antimicrobial MICs previously reported for these mutants. These results confirm that, in the 3 tested isolates with mutations that inactivate *ramR* or compromise its DNA binding, the enhanced expression of the *acrAB-tolC* efflux genes and the associated lower susceptibility to multiple antibiotics depend on a functional *ramA* gene.

In the 14028s reference strain and in the 2 quinolone-susceptible *S.* Typhimurium DT104 strains, the experimental inactivation of *ramR* resulted in increases in the transcripts levels of *ramA* (by 10–18-fold), *acrA* (by 2–3-fold), and *tolC* (by about 2-fold; Figure 1*B*). Phenotypically, this inactivation resulted in 2–4-fold increases in the MICs of the tested antimicrobials (Table 1). These results are consistent, in terms of magnitude of the regulation, with those obtained with the 2 clinical natural *ramR* mutants described above and their *ramR*-complemented derivatives.

We also tested the participation of *ramA* to the intrinsic level of resistance of the 14028s strain and of the DT104 1948SA96 strain. Inactivation of their *ramA* gene led to a 2-fold decrease in the MICs of most tested antimicrobials, including FQ (Table 1). This observation shows that RamA, even when expressed at a basal level under the repression by RamR, still contributes to the intrinsic level of resistance of these quinolone-susceptible isolates. For the 14028sΔ*ramA::kan* mutant, the MIC decreases were associated with lower levels of *acrA* and *tolC* transcripts (Figure 1*B*). However, this was not the case in the 1948SA96Δ*ramA::kan* mutant, whose levels of *acrA* and *tolC* transcripts were even higher than in the 1948SA96 parental isolate. Therefore, in the DT104 1948SA96 strain, the contribution of *ramA* to the intrinsic resistance phenotype could rely on a mechanism other than the positive regulation of the AcrAB-TolC efflux system. Additionally, deletion of the whole *ramRA* locus of this DT104 1948SA96 isolate resulted in the same MIC decreases as the *ramA* deletion, which also supports that *ramR* alteration can increase antimicrobial MICs only in the presence of a functional *ramA* gene.

Variable Effects of *ramRA* Mutations on the Expression of T3SS-1–Related Invasion Genes

Complementation of the 2 naturally *ramR*-mutated FQ-resistant isolates (DT204 strain 102SA00 and DT104 strain 543SA98) with a functional allele of *ramR* resulted in increases in transcript levels (by 4.5- and 12.1-fold, respectively) of the *sipA* gene coding for a secreted effector of the T3SS-1 (Figure 1*A*). However, only in 102SA00 was this increased

Downloaded from https://academic.oup.com/jid/article/207/5/794/1079166 by guest on 23 April 2024

sipA expression accompanied by increased expressions of the *hilA* and *invA* genes. These 2 genes encode the main SPI-1 positive regulator and an essential structural component of the T3SS-1, respectively. Therefore, in this 102SA00 strain *ramA* inactivation had an effect similar to that of the *ramR* complementation. These results indicate that in 102SA00 the *ramR* mutation is responsible for a lower expression of T3SS-1 invasion-related genes and that this effect is mainly mediated by the overexpression of *ramA*. In contrast, *ramA* inactivation in 543SA98 did not increase *sipA* expression to the same extent as complementation by a wild-type *ramR* (2.0-fold vs 4.5-fold, respectively). This suggests that *ramR* effect on invasion genes may be only partially mediated by *ramA* in this isolate. For the DT04 BN10055 strain mutated at the RamR binding site, complementation with the *ramR*-bearing plasmid had no significant effect on the expression of *hilA*, *invA*, and *sipA*. In contrast, *ramA* inactivation significantly increased the transcript levels of these genes (by 1.6-, 3.9-, and 8.7-fold for *hilA*, *invA*, and *sipA*, respectively). As was suggested by its location in the *ramA* promoter region, the mutation of strain BN10055 lowers the level of expression of the T3SS-1 invasion genes by a *ramA*-dependent pathway.

In the 14028s reference strain and in the 2 quinolone-susceptible *S.* Typhimurium DT104 strains, experimental inactivation of *ramR* resulted in decreased expressions of T3SS-1 genes, which is consistent with the complementation results described above. This effect was, however, different in magnitude between the strains (Figure 1*B*). It was the highest for the *ramR*-deleted mutant of the DT104 S/921495 isolate, whose transcript levels of the 3 T3SS-1 tested genes were reduced by 6–9-fold. In the 14028sΔ*ramR::kan* mutant, the transcript levels of these 3 genes were decreased by about 2-fold. In the 1948SA96Δ*ramR::kan* mutant, the transcript levels of *hilA* and *sipA* were reduced by 2.3- and 4.5-fold, respectively.

Inactivation of *ramA* showed no significant effect on invasion-related genes, except for a slight increase in transcript level (by 2.3-fold) of *sipA* for the 1948SA96Δ*ramA*::kan mutant. This increase was not observed in the *ramRA* deletion mutant of the same isolate. As suggested above for the DT104 543SA98 FQ-resistant clinical isolate, this observation raises the hypothesis that, in the DT104 1948SA96 strain, *ramR* positively influences the expression of invasion-related genes independently of *ramA*.

Effects of *ramRA* Mutations on Invasion of Intestinal Epithelial Cells

We used gentamicin protection assays to test whether the natural *ramRA* mutations of the 3 clinical isolates influenced their invasion phenotype. This was first performed in a Caco-2 intestine epithelial cells model. Introduction of a wild-type *ramR* gene increased the invasion level of the 102SA00 isolate by about 4-fold ($P = .002$), but this effect was not observed for the 2 other FQ-resistant isolates (Figure 2*A*). Additionally, strain 102SA00 and 2 derivatives (*ramR* complemented and *ramA* inactivated) were tested for their adhesion to and invasion of HT-29 cells, another line of intestinal epithelial cells (Figure 2*B*). Adhesion did not appear significantly different among these 3 strains. In contrast, both the *ramR*-complemented and the *ramA*-inactivated derivatives were 2.5-fold more invasive than the 102SA00 strain.

In accordance with these results, the *ramR*-complemented and *ramA*-inactivated derivatives of the 102SA00 isolate

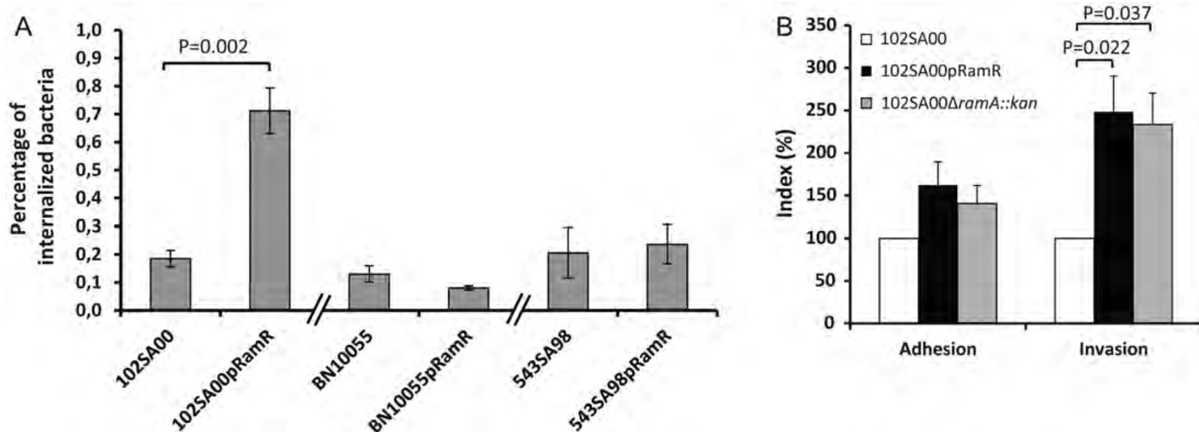

Figure 2. In vitro analysis of adhesion and/or invasion of intestinal epithelial cells by *ramRA* mutants. *A*, Invasion of Caco-2 cells by 3 isolates with natural mutations in *ramR* (543SA98 and 102SA00) or at the RamR-binding site (BN10055) and their *ramR*-complemented derivatives. Bars indicate the percentage of internalized bacteria ± standard error of the mean. *B*, Adhesion and invasion of HT-29 cells by the *ramR*-mutated 102SA00 isolate and its *ramR*-complemented and Δ*ramA::kan* derivatives. Results are expressed relative to values obtained for the 1002SA00 isolate, arbitrarily set at 100%. Bars indicate the percentages of attached or internalized bacteria ± standard error of the mean. When statistical significant differences were observed, the *P* value is indicated.

Downloaded from https://academic.oup.com/jid/article/207/5/794/1079166 by guest on 23 April 2024

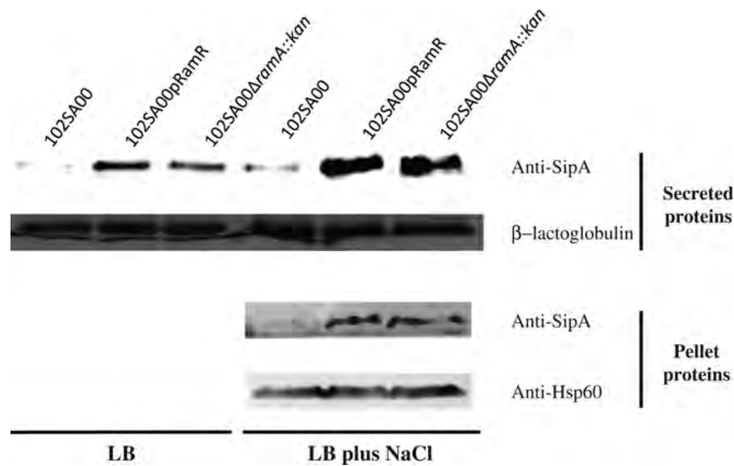

Figure 3. Western blot analyses of SipA proteins expressed and secreted by the 102SA00 isolate and its derivatives. Strains were either cultured in Luria-Bertani (LB) medium or in LB medium containing 0.3 M NaCl. Western blot against the constitutively expressed Hsp60 protein or the level of β-lactoglobulin in each sample detected after sodium dodecyl sulfate polyacrylamide gel electrophoresis migration and Coomassie brilliant blue G250 staining served as controls of loading onto the gel for pellet or secreted proteins respectively.

expressed and secreted a higher level of the SipA effector protein than their parental strain after culture in LB medium 0.3 M NaCl, conditions known to favor the transcription of T3SS-1 related genes (Figure 3). Similar results were observed for secreted proteins in regular LB medium. Data for SipA proteins present in the bacterial pellet after culture in LB broth are not presented because they were very hardly detected. All these results thus corroborated the increased level of *sipA* transcript found in 102SA00 derivatives (Figure 1*A*). Overall, this indicates that the natural *ramR* mutation in the DT204 strain 102SA00 decreases its invasiveness by a *ramA*-dependent mechanism, which is consistent with the results of T3SS-1 gene expression.

DISCUSSION

Antimicrobial resistance and virulence are 2 important bacterial factors that determine the clinical outcome of severe *Salmonella* infections. Therefore, existence of regulatory mechanisms acting on both resistance- and virulence-related genes is a matter of concern. As an example of a possible link, O'Regan et al have observed fitness costs, including reduced invasiveness in Caco-2 cells, for in vitro–selected ciprofloxacin-resistant *S.* Enteritidis mutants [4]. Previous studies have also reported a link between the AcrAB-TolC efflux system and pathogenicity [32–34]. A recent study by Bailey et al showed that, in addition to the regulation of AcrAB-TolC, RamA negatively influenced the expression of virulence genes in *S.* Typhimurium, including SPI-1 genes encoding structural proteins and effectors of the T3SS-1 [24]. This study was based on the single susceptible reference SL1344 strain and its experimental derivatives lacking a functional *ramA* or *ramR* gene or with a plasmid-mediated high-level expression of *ramA*. We decided to address, in the present study, whether these overlapping regulations of resistance and virulence traits also occurred in *S.* Typhimurium clinical isolates with different genetic backgrounds and showing resistance or decreased susceptibility to FQs. We used genetic complementation and experimental gene disruptions to study 3 MDR epidemic FQ-resistant strains bearing natural mutations in the *ramRA* locus and compare them with quinolone-susceptible strains of *S.* Typhimurium.

On the basis of data from the present and previous studies, the mechanism by which mutations in the *ramR* gene or at the RamR binding site located in the *ramA* operator region confer an increase in drug resistance levels via the increased expression of the *acrAB* and *tolC* efflux genes appears to be general among *S.* Typhimurium strains [17–23]. Moreover, this mechanism seems always dependent on a functional *ramA* gene.

In contrast, our results indicate that the effect of *ramRA* mutations on invasiveness is more complex and variable among strains. Indeed, the natural mutations that we studied negatively influenced the expression of T3SS-1 genes, although there were some differences between strains with regard to which genes were regulated, the magnitude of the regulatory effect, and the dependency of this regulation on a functional *ramA* gene. Bailey et al had noticed that a high-level (144-fold), plasmid-mediated, overexpression of *ramA* decreased the expression of many T3SS-1 genes, whereas the more modest (25-fold) *ramA* overexpression due to *ramR* disruption did not lead to a significant regulation of these genes. In the present study, among the 2 MDR FQ-resistant isolates carrying a natural *ramR* mutation, only 1 (DT204 strain

Downloaded from https://academic.oup.com/jid/article/207/5/794/1079166 by guest on 23 April 2024

102SA00) showed enhanced invasion ability after complementation with a wild-type *ramR* gene. This phenotypic effect correlated with important increases in the *hilA*, *invA*, and *sipA* transcript levels. The modest increase in the transcript level of the sole *sipA* gene in the *ramR*-complemented 543SA98 isolate may explain the absence of observable effect on the efficiency of invasion of Caco-2 cells. Yet, these 2 *ramR*-complemented isolates showed similar decreases in the *ramA* expression level (by 8-fold and 11-fold) as compared to their parental strain. This indicates that the regulatory effect of *ramR* mutations on the T3SS-1 genes is not determined only by the *ramA* overexpression level, but that it is also influenced by the genetic background of the strains. Furthermore, Bailey et al had observed that *ramA* disruption increased expression of SPI-1 genes in the susceptible *S.* Typhimurium SL1344 reference strain [24]. We did not observe any similar effect in the *ramA*-disrupted mutants derived from our susceptible control strains, which also reflects a variability in the regulation of T3SS-1 genes by *ramA* among *S.* Typhimurium strains.

The mechanism by which RamA represses SPI-1 genes is still unknown. However, RamA presumably acts only as a transcriptional activator, like the vast majority of other AraC/XylS family regulators [35]. In this hypothesis, the means by which it downregulates T3SS-1 genes is presumably indirect, via a regulatory input into SPI-1 that remains to be identified. The great complexity of the regulatory network of SPI-1 genes may offer more possibility for an interstrain polymorphism than the relatively simple and direct regulation of the AcrAB-TolC efflux system by the *ramRA* locus [26]. This may account for the observed variability of the effect of *ramRA* mutations on invasiveness, as opposed to the apparent constancy of their effect in terms of efflux-mediated decreased antimicrobial susceptibility.

In conclusion, the regulation of *S.* Typhimurium invasiveness by the *ramRA* locus appears variable both at the transcriptional and at the phenotypic level, even between strains of the same definitive phage type. Therefore, the acquisition of natural *ramRA* mutations conferring increased levels of resistance to multiple antibiotics and participating in FQ resistance may also negatively modulate the invasiveness of some, but not all, *S.* Typhimurium strains.

Notes

Acknowledgments. We are grateful to Isabelle Monchaux and Elisabeth Bottreau for excellent technical assistance.

Financial support. This work was supported by the Institut National pour la Recherche Agronomique (INRA) as an INRA-JSPS (Japan Society for the Promotion of Science) 2010–2012 joint research project, by the French Région Centre (grant 2008 00036085), and by the European Union with the European Regional Development Fund (grant 1634–32245).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed

References

1. Giraud E, Baucheron S, Cloeckaert A. Resistance to fluoroquinolones in *Salmonella*: emerging mechanisms and resistance prevention strategies. Microb Infect **2006**; 8:1937–44.
2. Velge P, Cloeckaert A, Barrow P. Emergence of *Salmonella* epidemics: The problems related to *Salmonella enterica* serotype Enteritidis and multiple antibiotic resistance in other major serotypes. Vet Res **2005**; 36:267–88.
3. Giraud E, Cloeckaert A, Baucheron S, Mouline C, Chaslus-Dancla E. Fitness cost of fluoroquinolone resistance in *Salmonella enterica* serovar Typhimurium. J Med Microbiol **2003**; 52:697–703.
4. O'Regan E, Quinn T, Frye JG, et al. Fitness costs and stability of a high-level ciprofloxacin resistance phenotype in *Salmonella enterica* serotype Enteritidis: reduced infectivity associated with decreased expression of *Salmonella* Pathogenicity Island 1 genes. Antimicrob Agents Chemother **2010**; 54:367–74.
5. Le Hello S, Hendriksen RS, Doublet B, et al. International spread of an epidemic population of *Salmonella enterica* Serotype Kentucky ST198 resistant to ciprofloxacin. J Infect Dis **2011**; 204:675–84.
6. Morita M, Hirose K, Takai N, et al. *Salmonella enterica* serovar Typhi in Japan, 2001–2006: emergence of high-level fluoroquinolone-resistant strains. Epidemiol Infect **2010**; 138:318–21.
7. Olsen SJ, DeBess EE, McGivern TE, et al. A nosocomial outbreak of fluoroquinolone-resistant *Salmonella* infection. N Engl J Med **2001**; 344:1572–9.
8. Xia SL, Hendriksen RS, Xie ZQ, et al. Molecular characterization and antimicrobial susceptibility of *Salmonella* isolates from infections in humans in Henan province, China. J Clin Microbiol **2009**; 47:401–9.
9. Collard JM, Place S, Denis O, et al. Travel-acquired salmonellosis due to *Salmonella* Kentucky resistant to ciprofloxacin, ceftriaxone and co-trimoxazole and associated with treatment failure. J Antimicrob Chemother **2007**; 60:190–2.
10. Weill FX, Bertrand S, Guesnier F, Baucheron S, Grimont PAD, Cloeckaert A. Ciprofloxacin-resistant *Salmonella* Kentucky in travelers. Emerg Infect Dis **2006**; 12:1611–2.
11. Sjolund-Karlsson M, Howie R, Rickert R, et al. Plasmid-mediated quinolone resistance among non-Typhi *Salmonella enterica* isolates, USA. Emerg Infect Dis **2010**; 16:1789–91.
12. Giraud E, Cloeckaert A, Kerboeuf D, Chaslus-Dancla E. Evidence for active efflux as the primary mechanism of resistance to ciprofloxacin in *Salmonella enterica* serovar Typhimurium. Antimicrob Agents Chemother **2000**; 44:1223–8.
13. Nikaido E, Yamaguchi A, Nishino K. AcrAB multidrug efflux pump regulation in *Salmonella enterica* serovar Typhimurium by RamA in response to environmental signals. J Biol Chem **2008**; 283:24245–53.
14. Nishino K, Nikaido E, Yamaguchi A. Regulation and physiological function of multidrug efflux pumps in *Escherichia coli* and *Salmonella*. Biochim Biophys Acta **2009**; 1794:834–43.
15. Baucheron S, Coste F, Canepa S, et al. Binding of the RamR repressor to wild-type and mutated promoters of the *ramA* gene involved in efflux-mediated multidrug resistance in *Salmonella enterica* serovar Typhimurium. Antimicrob Agents Chemother **2012**; 56:942–8.
16. Ricci V, Tzakas P, Buckley A, Coldham NC, Piddock LJV. Ciprofloxacin-resistant *Salmonella enterica* serovar Typhimurium strains are difficult to select in the absence of AcrB and TolC. Antimicrob Agents Chemother **2006**; 50:38–42.
17. Abouzeed YM, Baucheron S, Cloeckaert A. *ramR* mutations involved in efflux-mediated multidrug resistance in *Salmonella enterica* serovar Typhimurium. Antimicrob Agents Chemother **2008**; 52:2428–34.
18. Akiyama T, Khan AA. Molecular characterization of strains of fluoroquinolone-resistant *Salmonella enterica* serovar Schwarzengrund carrying multidrug resistance isolated from imported foods. J Antimicrob Chemother **2012**; 67:101–10.
19. Kehrenberg C, Cloeckaert A, Klein G, Schwarz S. Decreased fluoroquinolone susceptibility in mutants of *Salmonella* serovars other than Typhimurium: detection of novel mutations involved in modulated

Downloaded from https://academic.oup.com/jid/article/207/5/794/1079166 by guest on 23 April 2024

expression of *ramA* and *soxS*. J Antimicrob Chemother **2009**; 64: 1175–80.
20. Kim KY, Woo GJ. Expression of *acrB* and *ramA* in fluoroquinolone resistant mutants from multi-drug resistant *Salmonella enterica* serovar Haardt. Lett Appl Microbiol **2011**; 52:484–90.
21. O'Regan E, Quinn T, Pages JM, McCusker M, Piddock L, Fanning S. Multiple regulatory pathways associated with high-level ciprofloxacin and multidrug resistance in *Salmonella enterica* serovar Enteritidis: involvement of *ramA* and other global regulators. Antimicrob Agents Chemother **2009**; 53:1080–7.
22. Ricci V, Piddock LJV. Ciprofloxacin selects for multidrug resistance in *Salmonella enterica* serovar Typhimurium mediated by at least two different pathways. J Antimicrob Chemother **2009**; 63:909–16.
23. Zheng J, Cui SH, Meng JH. Effect of transcriptional activators RamA and SoxS on expression of multidrug efflux pumps AcrAB and AcrEF in fluoroquinolone-resistant *Salmonella* Typhimurium. J Antimicrob Chemother **2009**; 63:95–102.
24. Bailey AM, Ivens A, Kingsley R, Cottell JL, Wain J, Piddock LJV. RamA, a member of the AraC/XylS family, influences both virulence and efflux in *Salmonella enterica* serovar Typhimurium. J Bacteriol **2010**; 192:1607–16.
25. Zheng J, Tian F, Cui SH, Song J, Zhao S, et al. (2011) Differential gene expression by RamA in ciprofloxacin-resistant *Salmonella* Typhimurium. PLoS ONE **2011**; 6: e22161.
26. Golubeva YA, Sadik AY, Ellermeier JR, Slauch JM. Integrating global regulatory input into the *Salmonella* Pathogenicity Island 1 Type III secretion system. Genetics **2012**; 190:79–U497.
27. Baucheron S, Mouline C, Praud K, Chaslus-Dancla E, Cloeckaert A. TolC but not AcrB is essential for multidrug-resistant *Salmonella enterica* serotype Typhimurium colonization of chicks. J Antimicrob Chemother **2005**; 55:707–12.
28. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **2000**; 97:6640–5.
29. Fardini Y, Chettab K, Grepinet O, et al. The YfgL lipoprotein is essential for type III secretion system expression and virulence of *Salmonella enterica* serovar Enteritidis. Infect Immun **2007**; 75:358–70.
30. Amy M, Velge P, Senocq D, Bottreau E, Mompart F, Virlogeux-Payant I. Identification of a new *Salmonella enterica* serovar Enteritidis locus involved in cell invasion and in the colonisation of chicks. Res Microbiol **2004**; 155:543–52.
31. Rosselin M, Virlogeux-Payant I, Roy C, et al. Rck of *Salmonella enterica*, subspecies *enterica* serovar Enteritidis, mediates Zipper-like internalization. Cell Res **2010**; 20:647–64.
32. Nishino K, Latifi T, Groisman EA. Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. Mol Microbiol **2006**; 59:126–41.
33. Virlogeux-Payant I, Baucheron S, Pelet J, et al. TolC, but not AcrB, is involved in the invasiveness of multidrug-resistant *Salmonella enterica* serovar Typhimurium by increasing type III secretion system-1 expression. Int J Med Microbiol **2008**; 298:561–9.
34. Webber MA, Bailey AM, Blair JM, et al. The global consequence of disruption of the AcrAB-TolC efflux pump in *Salmonella enterica* includes reduced expression of SPI-1 and other attributes required to infect the host. J Bacteriol **2009**; 191:4276–85.
35. Tobes R, Ramos JL. AraC-XylS database: a family of positive transcriptional regulators in bacteria. Nucleic Acids Res **2002**; 30: 318–21.

Downloaded from https://academic.oup.com/jid/article/207/5/794/1079166 by guest on 23 April 2024