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RamA, a Member of the AraC/XylS Family, Influences Both Virulence and Efflux in *Salmonella enterica* Serovar Typhimurium▽†

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The transcriptomes of *Salmonella enterica* serovar Typhimurium SL1344 lacking a functional *ramA* or *ramR* or with plasmid-mediated high-level overexpression of *ramA* were compared to those of the wild-type parental strain. Inactivation of *ramA* led to increased expression of 14 SPI-1 genes and decreased expression of three SPI-2 genes, and it altered expression of ribosomal biosynthetic genes and several amino acid biosynthetic pathways. Furthermore, disruption of *ramA* led to decreased survival within RAW 264.7 mouse macrophages and attenuation within the BALB/c ByJ mouse model. Highly overexpressed *ramA* led to increased expression of genes encoding multidrug resistance (MDR) efflux pumps, including *acrAB*, *acrEF*, and *tolC*. Decreased expression of 34 *Salmonella* pathogenicity island (SPI) 1 and 2 genes, decreased SipC production, decreased adhesion to and survival within macrophages, and decreased colonization of *Caenorhabditis elegans* were also seen. Disruption of *ramR* led to the increased expression of *ramA*, *acrAB*, and *tolC*, but not to the same level as when *ramA* was overexpressed on a plasmid. Inactivation of *ramR* had a more limited effect on pathogenicity gene expression. *In silico* analysis of a suggested RamA-binding consensus sequence identified target genes, including *ramR*, *acrA*, *tolC*, *sipABC*, and *ssrA*. This study demonstrates that the regulation of a mechanism of MDR and expression of virulence genes show considerable overlap, and we postulate that such a mechanism is dependent on transcriptional activator concentration and promoter sensitivity. However, we have no evidence to support the hypothesis that increased MDR via RamA regulation of AcrAB-TolC gives rise to a hypervirulent strain.

Multidrug efflux pumps, such as the resistance-nodulation-division (RND) superfamily member AcrAB-TolC in the *Enterobacteriaceae*, export many substrates, including antibiotics (9, 27, 29, 49, 51, 64). AcrAB-TolC and homologous efflux systems are important in the pathogenicity of *Salmonella enterica* serovar Typhimurium (10, 14) and other bacterial species (11, 15, 16). Recent work by our laboratory in *S*. Typhimurium has shown that following the disruption of *acrA*, *acrB*, or *tolC*, a phenotype of decreased pathogenicity was associated with global changes in expression of genes involved in virulence (such as those in SPI-1), chemotaxis, and motility (70).

Regulation of RND efflux pumps has been widely studied in *Escherichia coli*, with the global AraC/XylS family regulators *marA*, *soxS*, and *rob* having been shown to activate expression of efflux pumps (5, 8, 46, 52, 71). Mechanistically, this is achieved via the binding, and subsequent activation, of discrete but degenerate nucleotide sequences within regulon genes, known as mar-, rob-, or soxboxes (30, 35, 42, 43). *E. coli marA* is part of the *marRAB* operon, linked to another operon, *marCD*, of unknown function. Expression of both is controlled from the same operator site, *marO* (20). Regulation of *E. coli marA* is provided through the local repressor *marR* (62), which controls the transcription of *marA* by binding to *marO*, preventing autoactivation of *marA*, which competes with *marR* for the marbox of *marO* (43). Expression of *marA* can be induced by various compounds and conditions, including exposure to bile salts (54), salicylate, cyclohexane, and the antibiotics tetracycline and chloramphenicol (65). The regulon of *marA* in *E. coli* comprises up to 60 genes, including *acrAB*, *tolC*, *ompF*, and *zwf* (4, 8, 27, 52). In *E. coli*, the level of *soxS* is controlled by the activator *soxR*, in contrast to the repressor activity of *marR* upon *marA*. Activation of *soxR* by superoxide stress induces expression of *soxS* and the subsequent induction of the *soxRS* regulon (23).

Salmonellae, *Klebsiella pneumoniae*, and *Enterobacter aerogenes* contain *ramA*, an additional member of the AraC/XylS family of transcriptional activators, which is absent from *E. coli* and *Shigella* spp. Overexpression of *ramA* confers multidrug resistance (6, 19, 28, 60, 67) through induction of *acrAB* and *tolC* (6). Regulation of *ramA* is provided locally by *ramR* (STM0580), whose function can be ablated by internal point mutations and insertions within the helix-turn-helix motif (3, 57) and/or deletions within the DNA-binding region between *ramR* and *ramA* (73). All such events confer multidrug resistance (MDR), presumably through the prevention of RamR binding to an operator sequence near *ramA*, and therefore the

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TABLE 1. Strains used in this study

S. Typhimurium strain code	Genotype	Description	Reference
L354	SL1344	Wild-type strain	72
L133	SL1344 *ramA*::*aph*	P22-transduced *ramA* disruptant	6
L786	L133 pTRC*hisA-ramA*	*ramA* under overexpression control of IPTG-inducible plasmid	6
L1007	SL1344 *ramR*::*aph*	P22-transduced *ramR* disruptant	58

subsequent relaxation of RamR repression at the *ramA* promoter (3, 57). This model is similar to the mechanism of repression of *E. coli marRA* (5, 62). Within *S*. Typhimurium, *marA*, *soxS*, and *rob* appear to play a lesser role in the regulation of MDR, and overexpression of *marA* and *soxS* is rarely observed within MDR clinical isolates (3, 29, 51). Furthermore, induction of *acrAB* by indole is regulated through *ramA*, independent of *marA*, *soxS*, or *rob* (48). Recently, we showed that inactivation of *acrB* or *tolC* was associated with overexpression of *ramA* (70). Prieto et al. (53) also showed that the presence or absence of an outer membrane protein in *S*. Typhimurium, AsmA, can affect *marA* expression. Taken together, these data suggest the existence of a feedback loop of regulation between AraC/XylS regulators such as *marA* and *ramA* and genes within their regulons.

In order to define the regulon of RamA, we investigated the effects of inactivation of *ramR* and the inactivation or artificial overexpression of *ramA* on the transcriptome of *S*. Typhimurium. Our data indicate that in addition to the regulation of multidrug efflux pump and accessory genes, expression of *ramA* influences expression of virulence genes, pathogenicity effectors, and genes within amino acid biosynthetic pathways.

MATERIALS AND METHODS

Strains and media used. Strains used in this study are listed in Table 1. All strains were derived from *S. enterica* serovar Typhimurium SL1344 (72). The *ramA*::*aph* disruptant was constructed as described previously (22) and then subsequently P22-transduced into SL1344 to avoid possible λ*red* recombinase-mediated mutations. To overexpress *ramA*, pTRC*hisA-ramA* (67) was transformed into L133 (*ramA*::*aph*). Ampicillin (Sigma-Aldrich; 50 μg/ml) was used in cultures to ensure retention of the plasmid, except during growth for RNA preparations for gene expression profiling. This was to prevent altered gene expression due to ampicillin exposure. pTRC*his-ramA* was not lost during this short (3-h) period (data not shown). Expression of *ramA* was quantified in both the presence and absence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), with IPTG induction giving a large increase in expression (6). Therefore, where *ramA* induction was required, sterile IPTG was added to the relevant growth media to a concentration of 1 mM. Luria-Bertani (LB) (Sigma-Aldrich) and MOPS (morpholinepropanesulfonic acid) minimal medium (Teknova Inc.) broth were used throughout this study.

RNA extraction and transcriptional analyses. Overnight cultures of *S.* Typhimurium SL1344 and the test strain were grown in MOPS minimal medium at 37°C as previously described (7). Microarray experiments were carried out exactly as described by Webber et al., (70) using the Pan-Salmonella Generation IV array generated at the Wellcome Trust Sanger Institute (Hinxton, United Kingdom). From each strain, three biological and two technical replicate RNA preparations were made. RNA was extracted and quantified as described previously (6, 70).

Transcriptomic experiments. RNA (25 μg) was used to generate probes labeled with either Cy-3 or Cy-5 (GE Healthcare) using Superscript III (Invitrogen). For each microarray experiment, the wild-type (SL1344) RNA was pooled after quantification to provide a common reference. Data were analyzed with Bioconductor (56) and Pathway Tools (37). Data with a *B* value (log odds value) of >0 and a *P* value of <0.05 were taken as significant.

Validation of microarray data. Reverse transcription PCRs (RT-PCRs) were performed as previously (6, 24) with a selection of SPI genes shown to be differentially expressed in the microarray experiments: *hilC*, *invF*, *sipC*, *sopB*, *ssrA*, and *ssaJ* (Table 3). Furthermore, expression of *ramA*, *acrB*, and *tolC* was also determined.

***In silico* analysis of putative rambox.** Nikaido et al. (48) described a putative degenerate rambox sequence, 5′ ATGGCACG[A/T]AA[A/C][A/G]CCAA[A/C][C/T][A/T] 3′. To establish whether this rambox was common to genes found to be differentially regulated by modification of *ramAR*, an *in silico* pattern search was performed against the *S*. Typhimurium LT2 genome (45) within xBase (17) and using the "fuzznuc" pattern search algorithm within EMBOSS (59). Given the degree of degeneracy known to be present in mar- and soxboxes (32, 44), various allowances of up to 6 mismatches were permitted and no restrictions were placed upon the search location. Data were cross-compared with transcriptomic data using the Microsoft Excel VLOOKUP function.

Protein purification and Western blotting of SipC. Protein purification and Western blotting were performed exactly as described previously (47, 70).

Adhesion and intracellular survival assays. Adhesion to and intracellular survival within eukaryotic macrophages (RAW 264.7) by *S*. Typhimurium SL1344 and strains derived therefrom were assessed as described previously (14). A two-tailed Student's *t* test was used to assess significance, using a *P* value of <0.05 as a cutoff.

Mouse model experiments. Experimental infections using the murine model of infection to compare SL1344 (wild type) and L133 (*ramA*::*aph*) were conducted essentially as previously described (39). Briefly, female 6- to 8-week-old BALB/c ByJ or C57/BL6 mice (five mice per group) were inoculated by the intragastric (i.g.) route with approximately 5×10^7 CFU of SL1344 or L133 (*ramA*::*aph*) in PBS (pH 7.4). Alternatively, mice were inoculated by the intravenous route with approximately 2.4×10^3 CFU of strain SL1344 or L133. Five days (i.g. inoculation) or four days (intravenous inoculation) after inoculation, mice were sacrificed and organs recovered by dissection. The number of CFU per organ was determined by plating serial 10-fold dilutions of homogenized tissue on LB agar plates or LB plus kanamycin (50 μg/ml). The statistical significance of differences in colonization of each organ was calculated using the Mann-Whitney test, using GraphPad Prism software. A *P* value of <0.05 was considered significant.

***Caenorhabditis elegans* survival assays.** *C. elegans* strain Bristol N2 was kindly donated by Robin May (University of Birmingham) and was cultured using standard methods as described previously (13, 61). Survival assays were performed essentially as previously (1, 2), with 20 larval stage 4 (L-4) *C. elegans* worms being picked and transferred onto each assay plate and with a minimum of 120 worms being used on three independent occasions. Plates were incubated at 25°C and scored daily for survival. No effects on worm survival were seen with IPTG, kanamycin, and ampicillin at the concentrations used in the experiments. Worms were transferred onto a fresh nematode growth medium (NGM) (33) plate containing the same bacterial test or control strain from the same original culture during the fertile period and reincubated at 25°C. A Kaplan-Meier estimate was used to determine the probability of *C. elegans* survival to the next day. Survival curves were generated by plotting probability of survival (*y*) against time (*x*). These curves were then compared using the log rank test to establish whether the difference between two curves was statistically significant, using a chi-squared test and a derived *P* value of <0.05 as the significance cutoff.

Microarray data accession numbers. Microarray datasets have been deposited in ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) with the following experiment identifiers: for *ramA*::*aph*, E-MEXP-2193; for *ramR*::*aph*, E-MEXP-2194; for pTRC*hisA-ramA*, E-MEXP-2195.

RESULTS

Inactivation of *ramA* and *ramR* and overexpression of *ramA* result in some common gene expression changes. Comparison of the transcriptome of L133 (*ramA*::*aph*) with that of SL1344 revealed that disruption of *ramA* resulted in significantly (log

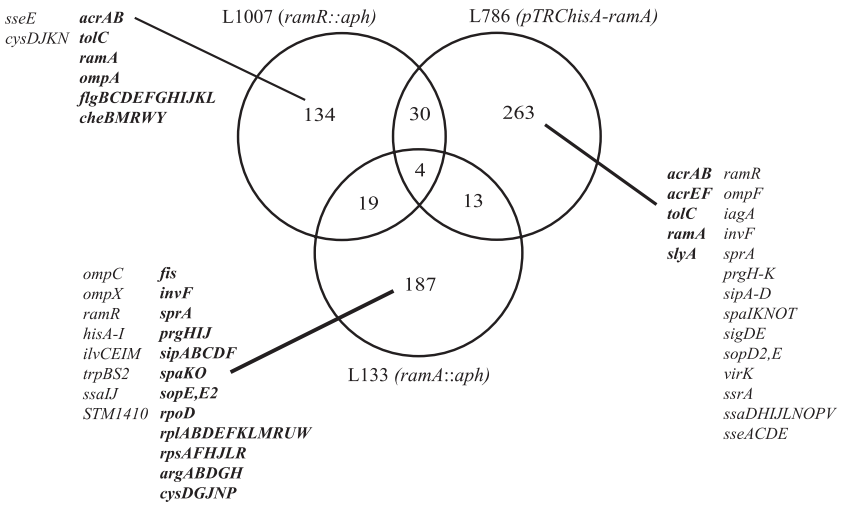

FIG. 1. Venn diagram of all significant ($B > 0$) gene expression changes within the comparison of L354 (*S*. Typhimurium SL1344) against L133 (*S*. Typhimurium *ramA*::*aph*), L1007 (*S*. Typhimurium *ramR*::*aph*), and L786 (*S*. Typhimurium pTRC*hisA-ramA*). Selected genes from each strain are displayed. Boldface shows increased gene expression; lightface shows decreased gene expression.

odds value > 0 and $P < 0.05$) altered expression of 223 genes (6% of the SL1344 genome). Of these 223, 119 genes were increased in expression and 104 were decreased in expression (Fig. 1; also, see Table S1 in the supplemental material). Comparison of the transcriptome of L1007 (*ramR*::*aph*) with that of SL1344 revealed that disruption of *ramR* resulted in significantly altered expression of 187 genes (5% of the SL1344 genome). Of these 187, 173 genes were increased in expression and 14 were decreased in expression (Fig. 1; also, see Table S2 in the supplemental material). Comparison of the transcriptome of L786 (L133 pTRC*hisA-ramA*) with that of SL1344 revealed that overexpression of *ramA* resulted in significantly altered expression of 310 genes (8% of the SL1344 genome). Of these 310, 158 genes were increased in expression and 152 were decreased in expression (Fig. 1; also, see Table S3 in the supplemental material).

Comparison of the transcriptomes of L133 (*ramA*::*aph*) and L1007 (*ramR*::*aph*) revealed 19 common genes, 16 of which were oppositely expressed (Fig. 1). Comparison of the transcriptomes of L133 and L786 (pTRC*his-ramA*) revealed 13 common genes; all are pathogenicity genes. Twelve were oppositely expressed, suggesting that they are directly affected by *ramA* expression (Fig. 1). Overexpression of *ramA* via inactivation of *ramR* (L1007), or artificially on pTRC (L786), showed that 30 genes were common to both. Of these, 23 were similarly increased or decreased in expression (Fig. 1). Four genes were significantly differentially expressed by all three strains; *ilvE*, *ilvM*, *napF*, and *pckA* (Fig. 1). Of these, two (*napF* and *pckA*) were increased in expression in all three strains, compared with SL1344. Two genes (*ilvE* and *ilvM*) were increased in expression when *ramR* was inactivated but decreased in expression when *ramA* was inactivated or overexpressed.

Overexpression of *ramA* and disruption of *ramRA* affect expression of genes encoding efflux pumps and porins. When *ramR* was inactivated, expression of *ramA* was increased 25-fold (Table 2). High-level overexpression in L786 (pTRC*hisA-ramA*) resulted in even greater expression (144.3-fold [Table 2]). These data were confirmed by RT-PCR (Fig. 2D) (6). After *ramR* disruption, expression of *acrA*, *acrB*, and *tolC* was increased 2.1- to 3.4-fold; even greater expression of these genes (5.2- to 8.7-fold [Table 2]) was seen when *ramA* was highly overexpressed. Increased expression of *acrB* and *tolC* was confirmed by RT-PCR (14.9-fold and 2.6-fold, respectively) (Fig. 2D) (6). Increased expression of genes encoding an additional RND efflux pump, AcrEF (2.5- and 3.0-fold, respectively [Table 2]) was also seen for L786 but not L133. Expression of *ramR*, the local repressor of *ramA*, was decreased 0.4-fold when *ramA* was highly overexpressed (Table 2), and expression of the porin gene *ompF* was decreased 0.2-fold (Table 2). No differential expression of the porin regulators *ompR* and *envZ* was observed.

RT-PCR revealed that upon inactivation of *ramA*, expression of *acrB* was decreased 0.2-fold compared to that in SL1344 (Fig. 2D). Furthermore, after *ramA* inactivation, expression of genes encoding the porins OmpC and OmpX was decreased (0.5- and 0.6-fold, respectively [Table 2]).

Inactivation of *ramA* increases expression of ribosomal biosynthetic genes and alters expression of genes encoding some amino acid biosynthetic pathways. Expression of 17 genes within the *rpl* and *rps* operons was statistically significantly increased after *ramA* inactivation (1.4- to 2.3-fold [Table 2]). Expression of 15 genes in the *arg*, *art*, and *cys* operons, responsible for arginine and cysteine biosynthesis, was also increased (4.2- to 12.1-fold [Table 2]). However, expression of 14 genes within the *his*, *ilv*, and *trp* operons, responsible for histidine, isoleucine, and tryptophan biosynthesis, respectively, was decreased (0.04- to 0.6-fold [Table 2]). Expression of genes encoding components of the glycolytic and gluconeogenic pathways, including *adh*, *zwf*, and *pckA*, was also affected by the disruption of *ramA*, with increased expression ($\leq$4.1-fold) and decreased expression ($\leq$0.2-fold) being observed (see Table S1 in the supplemental material). After disruption of *ramR*, expression of genes encoding proteins involved in cysteine biosynthesis, *cysCDHJKNU*, was statistically significantly decreased (0.3- to 0.6-fold [Table 2]).

TABLE 2. Genes encoding cell envelope,[a] ribosomal, and amino acid biosynthetic proteins

Gene or operon	Fold change in expression relative to SL1344[b]		
	L133	L786	L1007
Multidrug transporters/ regulators			
acrA	-	8.66	3.36
acrB	-	8.19	2.13
acrE	-	2.47	-
acrF	-	3.00	-
tolC	-	5.16	3.35
ompA	-	-	1.43
ompC	0.46	-	-
ompF	-	0.21	-
ompX	0.61	-	-
ramA	-	144.32	25.04
ramR	0.21	0.39	-
rpoD	1.36	-	-
Ribosomal biosynthesis			
rplA/B	1.98/1.65	-	-
rplD-F	1.44–1.84	-	-
rplK-M	1.74–2.04	-	-
rplR	2.33	-	-
rplU	1.80	-	-
rplW	1.82	-	-
rpsA	2.15	-	-
rpsF	1.97	-	-
rpsH	2.05	-	-
rpsJ	1.95	-	-
rpsL	2.08	-	-
rpsR	2.01	-	-
Amino acid biosynthesis			
argA/B	4.29/7.32	-	-
argD	12.09	-	-
argG/H	8.88/5.94	-	-
argR	4.16	-	-
aroA	0.47	-	-
aroH	0.16	-	-
artI/J	2.42/12.99	-	-
artP	3.25	-	-
cysC	-	-	0.42
cysD	11.32	-	0.25
cysG	3.75	-	-
cysH	-	-	0.53
cysJ	2.93	-	0.37
cysK	0.43	-	0.47
cysN	2.81	-	0.38
cysP	12.60	-	-
hisA-D	0.17–0.23	-	-
hisF-I	0.20–0.23	-	-
hisQ	2.72	-	-
ilvC	0.42	-	-
ilvE	0.64	-	-
ilvI	0.53	-	-
ilvM	0.54	-	-
trpB	0.11	-	-
trpS2	0.04	-	-

[a] Genes relevant to antibiotic transport and regulation thereof with significantly ($B > 0$) different expression compared with SL1344.
[b] -, no significant change in expression.

Disruption or high-level overexpression of *ramA* differentially affects expression of pathogenicity genes and proteins. The microarray experiments with L133 (*ramA*::*aph*) and L786 (L133 pTR*chisA-ramA*) revealed significantly altered expression of *Salmonella* pathogenicity island genes. Inactivation of *ramA* increased expression of 14 genes within SPI-1, including effectors such as *sipABCDF* and regulators such as *sprA* and *invF* (1.6- to 6.8-fold [Table 3]). Increased expression of *invF*, *sipC*, *sopB* and *ssrA* was confirmed by RT-PCR (Fig. 2A). No changes in expression of other known regulators of SPI-1, such as *phoPQ*, *pmrA*, and *hilC*, were seen; no differential expression of the latter was confirmed by RT-PCR (Fig. 2A). Expression of some genes within SPI-2, -4, -5, -6, and -7a was also altered in L133, with expression of genes from SPI-2, -6, and -7a (e.g., *ssaI* and STM1410) being decreased and that of genes within SPI-4 and -5 (e.g., *sigD* and *sopE*) being increased (Table 3). However, in contrast, when *ramA* was highly overexpressed (L786), decreased expression of 47 pathogenicity genes was observed, including many of the same SPI-1 genes for which increased expression upon *ramA* inactivation was seen; this was also confirmed by RT-PCR (Fig. 2B). Furthermore, RT-PCR validation also showed decreased expression of the regulatory gene *hilC* and the gene encoding the effector protein SopB (Fig. 2B). The RamA-dependent genes with decreased expression included those in SPI-1, -2, -4, -5, -6, and -7a as well as other genes whose products are also associated with the ability of salmonellae to be pathogenic (0.1- to 0.6-fold [Table 3]). Of interest, high-level overexpression of *ramA* in L786 was associated with increased expression of *slyA*, a known activator of SPI-2 (3.9-fold [Table 3]), despite the generally decreased expression of SPI-2 genes after *ramA* overexpression. Disruption of *ramR*, giving modest overexpression of *ramA*, altered expression of only one SPI-2 gene, *sseE*, which was decreased 0.51-fold (Table 3). However, RT-PCR validation revealed similarly (to high-level *ramA* overexpression) decreased expression of SPI genes (*invF*, *sopB*, *ssrA*, and *ssaJ*) (Fig. 2C).

To validate the decreased gene expression in L786, expression of SipC, a SPI-1 protein, was determined by Western blotting. No change in SipC production was observed for SL1344 or L133 (*ramA*::*aph*) (Fig. 3, lanes 1 and 3). As a control, IPTG was also added to both strains; no effect was seen upon SipC production (Fig. 3, lanes 2 and 4). L786 decreased production of SipC, in both the absence (0.76-fold) and presence (0.30-fold) of IPTG, compared to SL1344 (Fig. 3, lanes 5 and 6). Furthermore, in the absence of ampicillin, SipC production was decreased further; 0.24-fold (no IPTG) and 0.20-fold (with IPTG) (Fig. 3, lanes 7 and 8) compared to SL1344.

The ability of *S.* Typhimurium to adhere to and survive within RAW macrophages and to colonize mice and *C. elegans* is RamA dependent. As the expression of SPI-1 and SPI-2 genes was differentially altered by the overexpression and inactivation of *ramA*, and as production of SipC was decreased by overexpression of *ramA*, the ability of *ramA* mutants to adhere to and survive within RAW 264.7 macrophages and to colonize mice and *C. elegans* was investigated. Compared with SL1344, inactivation of *ramA* in L133 had no significant effect on adherence (Fig. 4A). The ability of L133 to survive within RAW 264.7 macrophages was significantly impaired, however (0.53-fold decrease [Fig. 4B]). Furthermore, L133 was recovered in significantly lower numbers (>100-fold difference in geometric mean) from the livers and spleens of BALB/c mice than the parental strain, following oral inoculation (Fig. 5A).

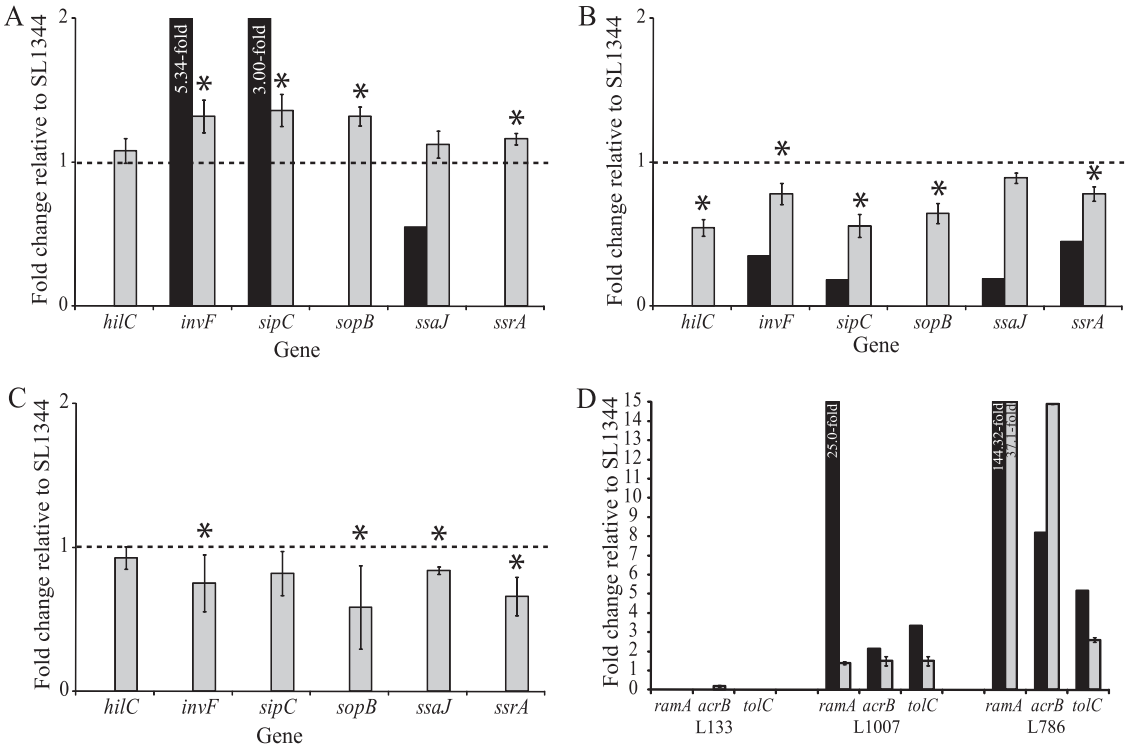

FIG. 2. RT-PCR for six SPI genes for *ramA*::*aph* (A), pTRC*hisA-ramA* (B), and *ramR*::*aph* (C) (asterisks show significant [$P < 0.05$] differences) and RT-PCR for *acrB*, *tolC*, and *ramA* (D) (all RT-PCR data shown are significant; $P < 0.05$). Black bars, microarray data; gray bars, RT-PCR data; dashed line, fold change of 1 (i.e., no change); for all array changes, a *B* value of >0 is considered significant.

The ability of this strain to colonize the livers and spleens of BALB/c mice following intravenous inoculation was not significantly different (Fig. 5B). These data were confirmed for the C57/BL6 mice (data not shown). When *ramA* was highly overexpressed in L786, the ability to adhere and survive within RAW 264.7 macrophages was significantly attenuated (Fig. 4). Unfortunately, in the mouse experiments, pTRC*hisA-ramA* could not be maintained, so the *C. elegans* model was used to further support the transcriptomic and cell culture data. In this model, L786 was significantly ($\chi^2 = 7.39$, $P = 0.0066$) attenuated in its ability to kill the worm compared to SL1344 (Fig. 6). L133 had no significant difference in pathogenicity compared to SL1344 ($\chi^2 = 1.83$, $P = 0.18$) (Fig. 6). Interestingly, even though limited SPI gene expression changes were observed for L1007, this strain was also significantly ($\chi^2 = 10.24$, $P = 0.0014$) attenuated compared to SL1344 (Fig. 6).

The SPI genes *pckA* and *ilvM* contain a rambox. *In silico* analyses using the degenerative rambox sequence proposed by Nikaido et al. (48) revealed several targets for RamA. These included *acrA* and *tolC*, whose expression has previously been shown to be increased by *ramA* overexpression (48) (Table 4). The SPI-1 effector genes *sipA*, *sipB*, and *sipC* and the SPI-1 regulator *invF* all contained a putative rambox, as did the SPI-2 gene *ssaJ* and the putative virulence effect gene *srfB* (Table 4). However, the expression of these RamA targets was variable, suggesting that RamA can activate or repress target gene expression. Only two genes containing a putative rambox, *pckA* and *ilvM*, were commonly expressed in all three strains (Table 4). The expression of *pckA*, which encodes the gluconeogenic pathway enzyme phosphoenolpyruvate carboxykinase, was increased in all three strains (1.6- to 2.3-fold), whereas the expression of *ilvM*, which encodes acetolactate synthase, was decreased in L133 (*ramA*::*aph*) and L786 (L133 pTRC*hisA-ramA*) 0.5- and 0.4-fold, respectively, but increased in L1007 (*ramR*::*aph*) (1.9-fold [Table 4]).

DISCUSSION

There is mounting evidence that RamA is a transcriptional activator that regulates genes conferring MDR in various species, including the salmonellae (6, 19, 28, 38, 48, 58, 67, 73). Therefore, we sought to resolve the regulon of *ramA* by comparing the transcriptomes of the wild-type SL1344 parental strain with those of three isogenic mutants in which *ramA* or *ramR* (the local repressor of *ramA*) was inactivated or in which *ramA* was highly overexpressed. Given that when genes which confer MDR, such as the those encoding the RND efflux pump AcrAB-TolC, are deleted, the virulence of the bacterium is attenuated (3, 14), it has been hypothesized that if overexpression of the same genes occurs, there may be a concurrent increase in virulence. Support for this hypothesis is derived from work with *Neisseria gonorrhoeae* (69, 68) showing that mutants overexpressing the Mtr efflux pump genes were more virulent than their wild-type parental strain.

As hypothesized, and confirming prior studies (3, 57), the microarray experiments revealed that inactivation of *ramR* resulted in overexpression of *ramA*. This is likely responsible for the increased expression of *acrAB* and *tolC* and decreased

TABLE 3. Pathogenicity genes and known regulators[a]

Gene/operon	Fold change in expression relative to SL1344[b]		
	L133	L786	L1007
Regulators of SPI-1			
fis	2.27	-	-
iagA	-	0.52	-
invF	5.34	0.35	-
sprA	3.73	0.34	-
SPI-1			
orgAa	1.64	0.59	-
prgH	4.61	0.50	-
prgI	3.24	0.34	-
prgJ	4.86	0.38	-
prgK	-	0.48	-
sicP	2.42	-	-
sipA	4.70	0.23	-
sipB	3.80	0.22	-
sipC	3.00	0.18	-
sipD	3.80	0.32	-
sipF	2.77	0.36	-
spaI	-	0.32	-
spaK	6.81	0.27	-
spaN	-	0.30	-
spaO	3.24	-	-
spaT	-	0.30	-
stpA	-	0.45	-
Regulators of SPI-2			
slyA	-	3.90	-
ssrA	-	0.45	-
SPI-2			
SL1323	-	0.57	-
ssaD	-	0.45	-
ssaH	-	0.49	-
ssaI	0.39	-	-
ssaJ	0.55	0.19	-
ssaL	-	0.42	-
ssaN	-	0.35	-
ssaO	-	0.34	-
ssaP	-	0.56	-
ssaV	-	0.43	-
sscA	-	0.25	-
sseA	-	0.25	-
sseC	-	0.26	-
sseD	-	0.27	-
sseE	-	0.25	0.51
STM1410	0.45	0.29	-
Other pathogenicity genes			
mig-14	-	0.53	-
pagC	-	0.14	-
sigD	3.17	0.18	-
sigE	-	0.19	-
sopD2	-	0.45	-
sopE	5.36	0.16	-
sopE2	4.76	0.16	-
STM0306	0.45	0.41	-
STM2780	0.51	0.19	-
STM4258	-	0.32	-
STM4260	3.48	0.39	-
STM4261	-	0.39	-
virK	-	0.36	-

[a] Genes with significantly ($B > 0$) changed expression in L133 (*ramA::aph*), L786 (L133 pTRC*hisA-ramA*), and L1007 (*ramR::aph*) compared with SL1344.
[b] -, no significant change in expression.

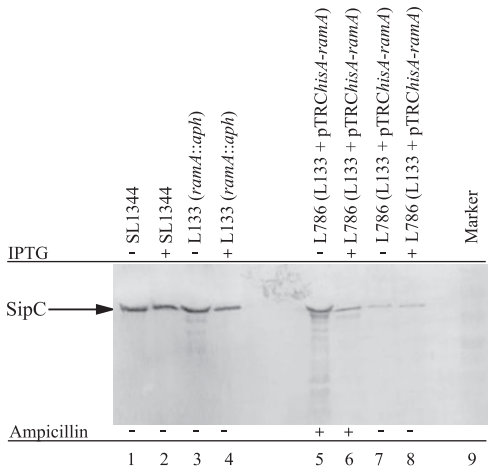

FIG. 3. Western blotting for SipC after growth of strains SL1344, L133, and L786 in minimal medium, in the presence and absence of IPTG (to induce overexpression of *ramA*) and ampicillin (to ensure maintenance of the pTRC plasmid).

expression of porin genes, such as *ompA*. As with overproduction of *E. coli marA* (21, 31), this gives rise to MDR due to reduced intracellular concentrations of antibiotic brought about by the decreased uptake of multiple agents and in-

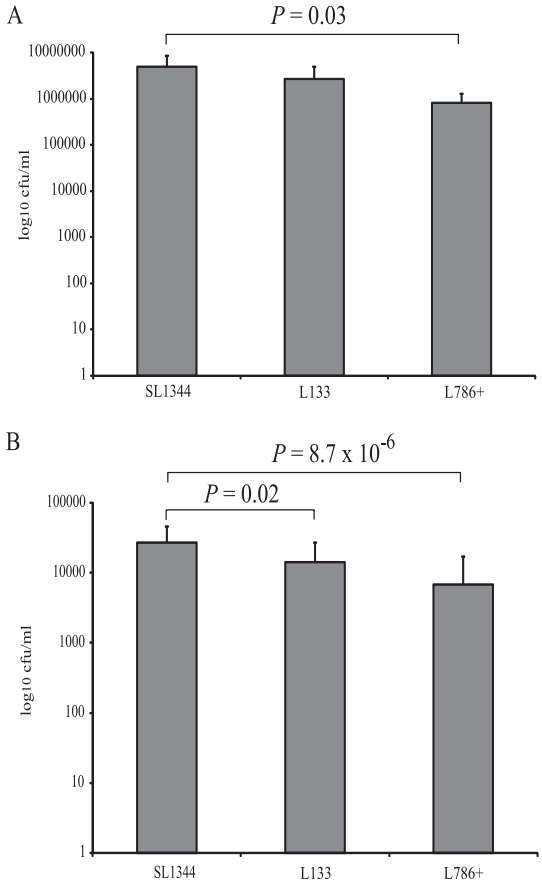

FIG. 4. Adhesion to (A) and survival in (B) RAW 264.7 mouse macrophages of SL1344, L133, and L786 (with IPTG).

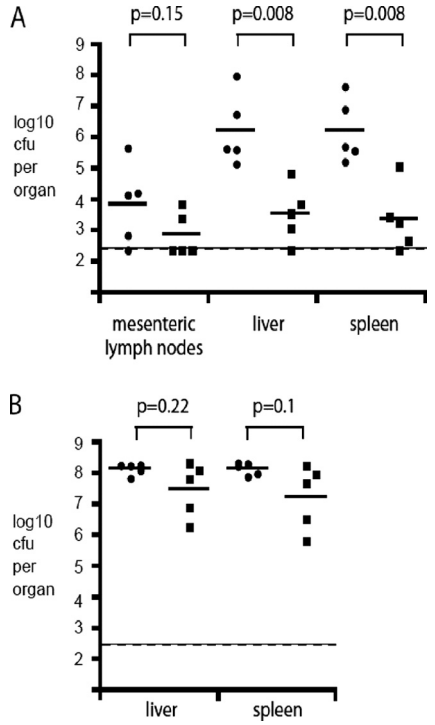

FIG. 5. Colonization of BALB/c mice by *S*. Typhimurium SL1344 and L133 (*ramA*::*aph*). Mice were inoculated orally with 4×10^7 CFU (A) or intravenously with 2.4×10^3 CFU (B). The CFU per organ of SL1344 (circles) and L133 (squares) and the geometric mean (horizontal bar) are indicated. *P* values were calculated using the Mann-Whitney test.

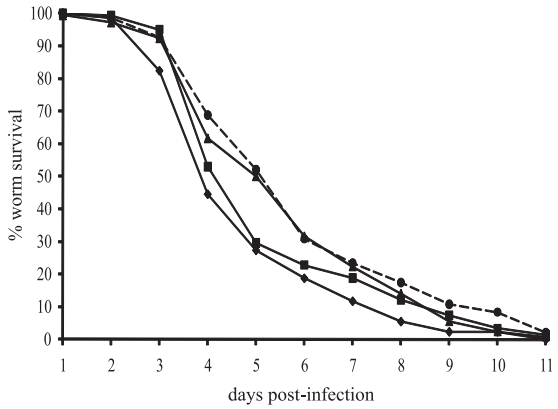

FIG. 6. Survival of *Caenorhabditis elegans* after infection with SL1344 (diamonds), L133 (squares), L1007 (circles), or L786 (triangles).

creased drug efflux. The magnitude of increased expression of *acrAB* and *tolC* was greater when *ramA* was highly overexpressed through an IPTG-inducible plasmid, which in addition increased expression of genes encoding an additional efflux pump, AcrEF, and the porin gene *ompF*. These data suggest an intracellular concentration-dependent response of the bacterium to increasing overexpression of *ramA*/RamA. We postulate that such a mechanism is dependent on transcriptional activator concentration and promoter sensitivity, similar to that established previously for *E. coli marA*, *soxS*, and *rob* (41). Our data further suggest that bacterial cells carefully "orchestrate" the level of RamA, with too much or none being deleterious to the bacterium. This is reminiscent of levels of the PhoP/Q system, where expression of these genes and those within its regulon are produced, and at the correct level, only under the appropriate conditions (50). Conversely, inactivation of *ramA* affected the expression of very few drug efflux pump genes, and *ompC*, rather than *ompF*, was decreased. These data lend further weight to the hypothesis that RamA regulates expression of MDR genes encoding porins and efflux pumps.

Nikaido et al. (48) showed the presence of a putative rambox in *acrAB* and *tolC*. In this study, we found that *ramR* also contains a rambox, and our data support previous hypotheses that *ramR* acts as a local repressor of *ramA* (3, 57). We also hypothesize that the absence of functional RamA reduces the expression of *ramR* in a mechanism aimed to restore wild-type expression of *ramA*, whereas overexpression of *ramA* successfully competes with RamR and suppresses further expression of *ramR*. The *ramR*-independent mechanism by which *ramA* expression is returned to basal levels is unclear. However, our data support the hypothesis that *ramRA* works through a mechanism similar to that of *marRA* within *E. coli*, with RamA being able to compete with RamR in binding to a hypothetical operator sequence *ramO*, leading to autoinduction of self-expression. Further work is required in order to elucidate the exact composition of any "*ramO*" sequence within *ramR*, as a significant amount of degeneracy (6 base mismatches) was used to generate the sequence of the rambox used in this study. Putative ramboxes were also identified within the pathogenicity effector genes *sipABC* and *ssaJ* and the AraC/XylS family SPI regulator *invF*. This coincided with the decreased expression of SPI-1 and SPI-2 genes upon high-level *ramA* overexpression, while *ramA* disruption induced SPI-1 expression. Previous work with AraC/XylS DNA regulators has suggested that these are transcriptional activators (55, 66), whereas in our study RamA appeared to repress the expression of *invF*, *ssrA* and *sprA*, all additional AraC/XylS family regulators. The association of an AraC/XylS-family DNA regulator with control of virulence genes has been reported previously for InvF and ToxT (34, 36), but purely as an activator of further genes. This suggests that the general downregulation of many SPI-1 and SPI-2 genes may not be due to direct interaction with RamA but may be due to the downregulation of *invF* and *ssrA*. The concentration-dependent regulation of the *ramA* regulon also appeared to extend to genes involved in mediating the pathogenicity of *S*. Typhimurium, as inactivation of *ramR*, and therefore lower-level overexpression of *ramA*, did not significantly affect the expression of SPI genes.

Given that disruption of *ramA* led to differential pathogenicity gene regulation, we assessed the effect(s) upon pathogenicity within various models. Overexpression of *ramA* decreased expression of SPI-1 and SPI-2 genes, decreased production of SipC, decreased adhesion and survival within RAW 264.7 macrophages, and attenuated pathogenicity within the *C. elegans* worm model. These findings suggest that RamA overexpression not only affects expression of efflux pumps but also affects the pathogenicity of *S*. Typhimurium, via altered SPI gene expression. Expression of *slyA*, a known regulator of

TABLE 4. Selected genes commonly differentially regulated in two or more arrays which contain a putative rambox

Gene	Product	Fold change[a]		
		L133 (*ramA*::*aph*)	L1007 (*ramR*::*aph*)	L786 (L133 pTR*ChisA-ramA*)
acrA	Multidrug resistance accessory protein	-	3.36	8.66
ilvM	Acetohydroxy acid synthase II, small subunit	0.54	1.87	0.37
invF	Possible AraC-family regulatory protein	5.34	-	0.35
pckA	Phosphoenolpyruvate carboxykinase	2.31	1.60	2.27
ramR	Putative *tetR*-family transcriptional regulator	0.21	-	0.39
sipA	Pathogenicity island 1 effector protein	4.70	-	0.23
sipB	Pathogenicity island 1 effector protein	3.80	-	0.22
sipC	Pathogenicity island 1 effector protein	3.00	-	0.18
srfB	Putative virulence effector protein	-	1.93	0.62
ssaJ	Putative pathogenicity island lipoprotein	0.55	-	0.19
tolC	Outer membrane protein	-	3.35	5.16

[a] Relative to *S.* Typhimurium SL1344. -, no significant change.

SPI-2 (40), was increased upon overexpression of *ramA* and in opposition to the expression of the pathogenicity island genes it regulates (26). These observations again suggest that some of the transcriptional changes that appear to be RamA dependent may be related to the levels of other regulators. Furthermore, these data suggest that the intracellular concentration of RamA defines not only the scope of its regulon but also the level of differential expression of targets within it. Disruption of *ramR*, leading to modest overexpression of *ramA*, also decreased expression of pathogenicity genes, though for far fewer genes than seen with plasmid-driven *ramA* overexpression. Given the attenuation of L1007 within the *C. elegans* model, this again supports our hypothesis that *ramA*/RamA expression influences not only efflux pump gene expression but also pathogenicity.

Less clear is the effect upon pathogenicity after *ramA* disruption. As increased expression of a large number of SPI-1 genes was observed, it was anticipated that increased adhesion to the mouse macrophage would be observed. However, no effect upon adhesion to macrophages or attenuation within the *C. elegans* model was observed. Interestingly, attenuation in the ability to survive both within the macrophage and within the mouse model was seen. This attenuation may suggest that the decreased expression of only three SPI-2 genes plays a greater role in defining pathogenicity in these two models than the increased expression of many more genes in SPI-1. This is supported by the lack of attenuation within the *C. elegans* of the *ramA*::*aph* strain. Prior work with *C. elegans* (2) has shown that salmonella does not cause an intracellular infection, and our data and those of others (63) suggest that adhesion, rather than intracellular survival, is measured within this model. This also correlates with recent work in salmonella that demonstrated that SPI-1 genes are less critical for pathogenicity within the mouse systemic infection model (18) than SPI-2 genes.

Inactivation of *ramA* revealed large changes in expression of ribosomal, glycolytic/gluconeogenic, and amino acid biosynthetic pathways, whereas overexpression of *ramA* had little effect on these genes. This could be due to the increased expression of SPI-1 genes prompting cellular metabolism to switch to an intracellular-adapted form. Bowden et al. (12) showed that glycolysis and glucose transport are important in the infection of mouse macrophages (12). Furthermore, transcriptomic analyses by Eriksson et al. (25) of *S.* Typhimurium grown intracellularly within macrophages revealed that the expression of 20 to 30% of genes in ribosomal and amino acid biosynthetic pathways was affected by intracellular growth, with an approximately equal split between increased and decreased expression. Within the subset of amino acid biosynthetic genes whose expression was altered after intracellular macrophage growth (25), cysteine biosynthesis was generally increased. Within this study, expression of cysteine biosynthetic genes was generally increased after inactivation of *ramA*, but decreased after inactivation of *ramR*. This lends further support to the hypothesis that RamA represses expression of genes contributing to pathogenicity and intracellular survival genes. Further interrogation of data obtained by Eriksson et al. (25) revealed that expression of *ramA* was increased 4, 8, and 12 h after macrophage infection between 7.6- and 24.3-fold, again suggesting a role for RamA in the control of salmonella pathogenicity. However, expression of *ramR* was relatively constant compared to the control, increasing to a maximum of only 1.5-fold.

In this study, we show that production of AcrAB-TolC and proteins that confer the ability for *Salmonella enterica* serovar Typhimurium to be pathogenic is RamA dependent. This demonstrates that the regulation of a mechanism of MDR and expression of virulence genes show considerable overlap. However, we have no evidence to support the hypothesis that increased drug resistance via AcrAB-TolC and RamA production gives rise to a hypervirulent strain.

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