

## *ssrA* (tmRNA) Plays a Role in *Salmonella enterica* Serovar Typhimurium Pathogenesis

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*Escherichia coli ssrA* encodes a small stable RNA molecule, tmRNA, that has many diverse functions, including tagging abnormal proteins for degradation, supporting phage growth, and modulating the activity of DNA binding proteins. Here we show that *ssrA* plays a role in *Salmonella enterica* serovar Typhimurium pathogenesis and in the expression of several genes known to be induced during infection. Moreover, the phage-like attachment site, *attL*, encoded within *ssrA*, serves as the site of integration of a region of *Salmonella*-specific sequence; adjacent to the 5' end of *ssrA* is another region of *Salmonella*-specific sequence with extensive homology to predicted proteins encoded within the unlinked *Salmonella* pathogenicity island SPI4. *S. enterica* serovar Typhimurium *ssrA* mutants fail to support the growth of phage P22 and are delayed in their ability to form viable phage particles following induction of a phage P22 lysogen. These data indicate that *ssrA* plays a role in the pathogenesis of *Salmonella*, serves as an attachment site for *Salmonella*-specific sequences, and is required for the growth of phage P22.

Many virulence functions that contribute to in vivo fitness are encoded on pathogenicity islands, sequences presumed to have been acquired by horizontal transfer as evidenced by their atypical base composition and codon usage (17). We have recently used in vivo expression technology (20, 34, 35) to identify several *Salmonella enterica* serovar Typhimurium in vivo-induced (*ivi*) acquired sequences, which are distinct within and between *Salmonella* serovars and contribute to fitness within the host (10). One of these acquired virulence regions, *iviXXII*, is composed of a mosaic of *Salmonella*-specific sequences that is also serovar specific (present in a distinct set of serovars of *Salmonella* but not in *E. coli* or several other enteric pathogens). Removal of a 17-kb portion of the *iviXXII* region conferred a greater than 200-fold defect in virulence in a murine model of typhoid fever (10).

*ssrA* resides in or near the junction point of this mosaic of *Salmonella*-specific sequence (10) and is also known to serve as the insertion site for acquired sequences such as the cryptic phage CP4-57 in *Escherichia coli* (27) and pathogenicity islands in *Vibrio cholerae* (25, 30) and *Dichelobacter nodosus* (2), the causative agents of cholera and ovine foot rot, respectively. *ssrA* encodes a small, stable RNA molecule (~350 nucleotides), tmRNA, which may serve as both a tRNA and an mRNA, tagging partially synthesized proteins for degradation (26). Additionally, tmRNA plays other diverse roles in *E. coli* including regulating an alternative protease activity (27), modulating DNA binding protein activity (41), and contributing to the growth of  $\lambda$ -P22 hybrid phage (40, 55). We investigated whether *ssrA* contributed to the pathogenesis of *Salmonella* since *ssrA* mapped within a 17-kb region that is required for pathogenesis (10) and RNA molecules are also known to play a key role in the virulence of many organisms (17, 39, 43).

### MATERIALS AND METHODS

**Bacterial strains and phage.** All *S. enterica* serovar Typhimurium strains used in this study were derived from strain ATCC 14028 (CDC 6516-60) and are listed in Table 1. The high-frequency generalized transducing bacteriophage P22 mutant HT105/1, *int-201*, was used for all transductional crosses (47), and phage-free, phage-sensitive transductants were isolated as previously described (5). The isolations of all *ivi* fusions used in this study were described previously (19). Bacteriophage P22-3657 and *S. enterica* serovar Typhimurium LT2 strain MST3357 were kindly provided by Stan Maloy. P22-3657 contains a deletion in gene 9, encoding phage P22 tail protein (15). MST3357 constitutively expresses P22 tail protein, which is necessary for P22-3657 to form plaques.

**Nomenclature.** The nomenclature is generally as described previously (4, 7, 13). Unless otherwise specified, the mutation designations used in this study are those of Sanderson et al. (45). The nomenclature z--::Tn10 refers to a Tn10 insertion in a "silent" DNA region; the z-- describes the map position of the insertion (44). The nomenclature used for chromosomal rearrangements is as described previously (8, 21, 33, 48).

**Media and chemicals.** Luria broth (LB) (11) was the laboratory medium used in this study except for  $\beta$ -galactosidase assays, for which 2-(*N*-morpholino)ethanesulfonic acid (MES) buffered to pH 5.5 and supplemented with 0.05 mM Mg<sup>2+</sup> was used. Unless otherwise specified, the final concentrations of antibiotics were as follows: ampicillin, 50  $\mu$ g/ml (LB) or 16  $\mu$ g/ml (MES); kanamycin, 50  $\mu$ g/ml; and tetracycline, 20  $\mu$ g/ml. Mitomycin C and *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) were purchased from Sigma.

**Construction of *ssrA* mutations. (i) Kn<sup>r</sup> deletions.** Deletions within the *ssrA* region were constructed by a PCR-based strategy as previously described (23). The resulting mutations have defined deletion endpoints that contain a Kn<sup>r</sup> (kanamycin) determinant at the deletion joint point. The endpoints and primers used to generate the deletions were as follows: MT2055, 5'-TTTGATCGCGG GAAACAC-3' and 5'-GCAATGCACCTTGGGTTT-3'; MT2094, 5'-TACTCA TCTCCTGAGTGG-3' and 5'-GCAATGCACCTTGGGTTT-3'; MT2095, 5'-GCGAGCGGTAAATCGTTG-3' and 5'-CGGTGTATCAGGCTATCG-3'; MT2096, 5'-GAATGCGCAGTTAACGGC-3' and 5'-CGATAGCCTGATACA CCG-3'; and MT2194, 5'-TTTCGGACGCGGGTTCAA-3' and 5'-GCAATGC ACCTTGGGTTT-3'.

**(ii) Tn10d-Tc insertions.** Tn10d-Tc insertions in the *ssrA* region were isolated using localized mutagenesis to a genetically linked *ivi* fusion, *iviXXII* (10). Briefly, MT1219 (*iviXXII*) was used as a recipient of phage P22 grown on a genomic pool of Tn10d-Tc insertions. Tetracycline-resistant transductants (Tc<sup>r</sup>) were selected and screened for loss of *lacZ* (inherent in *ivi* fusions) on LB plates containing X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside). These Lac<sup>-</sup> Tn10d-Tc insertions were pooled, and a P22 lysate grown on these insertions was used to transduce *S. enterica* serovar Typhimurium harboring a recombinant plasmid containing a 3.4-kb DNA insert of the *ssrA* region (cloned within pWKS30 [53], Ap<sup>r</sup>) to tetracycline resistance. The Tc<sup>r</sup> transductants were pooled, and plasmid DNA was isolated and used to electroporate *E. coli* DH5 $\alpha$  *lpir* to tetracycline resistance. These Tc<sup>r</sup> Ap<sup>r</sup> transformants contain Tn10d-Tc insertions in the 3.4-kb *ssrA* region. Transposon insertion joint points on the plasmid were determined by sequence analysis using a primer (5'-ACCTTGGTCAACCAACGC-3')

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TABLE 1. Bacterial strains and phage used in this study

Strain or phage <sup>a</sup>	Genotype	Source or reference
<i>Salmonella</i> strains		
ATCC 14028	Wild type	CDC 6516-60
MT1219	DUP3133 [ $\phi$ ( <i>iviXXII-purA</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET1* ( <i>iviXXII</i> <sup>+</sup> )]	19
MT1780	DUP3458 [ $\phi$ ( <i>vacB</i> <sup>-</sup> - <i>purA</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET* ( <i>vacB</i> <sup>+</sup> )]	19
MT2055	$\Delta$ <i>zff7503</i> ::Kn (17-kb deletion of <i>ssrA</i> region)	10
MT2057	<i>zjf7504</i> ::MudJ (33% linked to <i>purA</i> )	10
MT2094	$\Delta$ <i>zff7505</i> ::Kn (3.4-kb deletion removing <i>ssrA</i> , Slp, and Orf)	This work
MT2095	$\Delta$ <i>zff7506</i> ::Kn (2.0-kb deletion removing HlyB and HlyD)	This work
MT2096	$\Delta$ <i>zff7507</i> ::Kn (3.0-kb deletion removing HlyB and AggA)	This work
MT2121	<i>ssrA1</i> ::Tn10d-Tc	This work
MT2122	<i>zff7508</i> ::Tn10d-Tc (within SlpA)	This work
MT2123	<i>zff7509</i> ::Tn10d-Tc (intergenic between SlpA and Orf)	This work
MT2124	<i>zff7510</i> ::Tn10d-Tc (within Orf)	This work
MT2194	$\Delta$ <i>ssrA2</i> ::Kn	This work
MT2207	P22-3657 lysogen in 14028	This work
MT2208	P22-3657 lysogen in <i>ssrA1</i> ::Tn10d-Tc	This work
MT2218	DUP3122 [ $\phi$ ( <i>asnS</i> <sup>-</sup> - <i>purA</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET1* ( <i>asnS</i> <sup>+</sup> )]	19
MT2219	DUP3229 [ $\phi$ ( <i>thiI</i> <sup>-</sup> - <i>purA</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET1* ( <i>thiI</i> <sup>+</sup> )]	19
MT2220	DUP3458 [ $\phi$ ( <i>vacB</i> <sup>-</sup> - <i>purA</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET1* ( <i>vacB</i> <sup>+</sup> ) $\Delta$ <i>ssrA2</i> ::Kn]	This work
MT2221	DUP3122 [ $\phi$ ( <i>asnS</i> <sup>-</sup> - <i>purA</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET1* ( <i>asnS</i> <sup>+</sup> ) $\Delta$ <i>ssrA2</i> ::Kn]	This work
MT2222	DUP3229 [ $\phi$ ( <i>thiI</i> <sup>-</sup> - <i>purA</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET1* ( <i>thiI</i> <sup>+</sup> ) $\Delta$ <i>ssrA2</i> ::Kn]	This work
MT2231	DUP3360 [ $\phi$ ( <i>f304-cat</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET8* ( <i>f304</i> <sup>+</sup> )]	19
MT2232	DUP3136 [ $\phi$ ( <i>dedE-purA</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET1* ( <i>dedE</i> <sup>+</sup> )]	19
MT2234	DUP3448 [ $\phi$ ( <i>yoaA-cat</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET8* ( <i>yoaA</i> <sup>+</sup> )]	19
MT2235	DUP3360 [ $\phi$ ( <i>f304-cat</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET8* ( <i>f304</i> <sup>+</sup> ) $\Delta$ <i>ssrA2</i> ::Kn]	This work
MT2236	DUP3136 [ $\phi$ ( <i>dedE-purA</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET1* ( <i>dedE</i> <sup>+</sup> ) $\Delta$ <i>ssrA2</i> ::Kn]	This work
MT2238	DUP3448 [ $\phi$ ( <i>yoaA-cat</i> <sup>+</sup> - <i>lacZ</i> <sup>+</sup> <i>lacY</i> <sup>+</sup> )* pIVET8* ( <i>yoaA</i> <sup>+</sup> ) $\Delta$ <i>ssrA2</i> ::Kn]	This work
MST3357	LT2 <i>leuA414</i> (Am) <i>supE</i> Fels <sup>-</sup> /pJS28 (P22 gene 9/Ap <sup>r</sup> )	Stan Maloy
<i>E. coli</i> strain		
DH5 $\alpha$ $\lambda$ pir	F <sup>-</sup> <i>endA1 recA1 hsdR17</i> ( $r_K^- m_K^+$ ) <i>supE44 thi-1 gyrA96 relA1</i> $\phi$ 80 <i>dlacZM15</i> $\Delta$ ( <i>lacZYA-argF</i> )U169 $\lambda$ pir	18, 29
Phage		
P22 HT 105/1 HT 105/1 <i>int-201</i>		47
P22-3657	Kn6 P <sub>ant</sub> $\downarrow$ -11c <i>arc-amH1605</i> P <sub>sar</sub> $\downarrow$ <i>subS lac2 9-delTB</i>	Stan Maloy

<sup>a</sup> All bacterial strains with an MT designation are derivatives of *S. enterica* serovar Typhimurium ATCC 14028 (CDC 6516-60). MST3357 is a derivative of *S. enterica* serovar Typhimurium strain LT2.

which hybridizes to the right end of Tn10d-Tc. To move these Tn10d-Tc<sup>r</sup> transposon insertions into the *S. enterica* serovar Typhimurium chromosome, phage P22 was grown on the strains containing the Tn10d-Tc-bearing plasmids and used to transduce wild-type *S. enterica* serovar Typhimurium 14028 to tetracycline resistance. Transductants containing a Tn10d-Tc insertion which had recombined into the chromosome were confirmed by screening for Ap<sup>s</sup> (loss of pWKS30) and by verifying linkage to the parental *ivi* fusion, *iviXXII*.

**Competitive index assay in BALB/c mice.** Mutant and wild-type cells were grown overnight in LB medium. BALB/c mice were intraperitoneally infected with a total of 10<sup>3</sup> cells of a 1:1 ratio of mutant to wild type (MT2057). The mice were sacrificed after 5 days, and the bacterial cells were recovered from the spleen; the competitive index is the ratio of the number of mutant to wild-type bacteria recovered.

**Phage P22 assays. (i) EOP.** To determine the relative efficiency of plating EOP for *ssrA* strains, serial dilutions of phage P22 (HT105/1 *int-201*) were mixed with wild-type and *ssrA* strains and plated on LB medium containing 0.75% top agar. The number of PFU was determined on both wild-type and *ssrA* hosts. The EOP was calculated by dividing the number of PFU per milliliter obtained on the *ssrA* host strain by the number of PFU per milliliter obtained on the wild-type host.

**(ii) Phage adsorption.** Phage P22 adsorption assays were performed by adding approximately 10<sup>8</sup> overnight-grown bacteria and 10<sup>5</sup> phage P22 to an Eppendorf tube containing prewarmed LB medium (37°C) in a total volume of 1 ml. A no-cell control was used to calculate the efficiency of adsorption. After a 15-min incubation, the cells were centrifuged at low speed for 30 s, and then at full speed spin 90 s, after which the supernatant was immediately transferred to a fresh tube. To determine the remaining PFU, supernatants were subjected to titer determination on wild-type *S. enterica* serovar Typhimurium. The percent adsorption is calculated as follows: [(number of phage PFU recovered from the supernatant of phage without cells - number of PFU recovered from the supernatant of phage with cells)/number of phage PFU recovered from the supernatant of phage without cells]  $\times$  100.

**(iii) Lysogen formation.** The frequency of P22 lysogen formation was determined as follows. Overnight cultures of wild-type and *ssrA* *S. enterica* serovar

Typhimurium were diluted 1:50 and incubated for 2.5 h at 37°C. At this point, the number of viable cells was determined and 4  $\times$  10<sup>7</sup> bacteria were mixed with 1.5  $\times$  10<sup>9</sup> phage P22-3657 (which contains a kanamycin resistance gene in place of the phage *mnt* gene [15]), for a multiplicity of infection (MOI) of approximately 40, in a total volume of 0.4 ml. Following a 1-h standing incubation at 37°C, the cell-phage mixture was serially diluted and plated on LB plates containing kanamycin to select for lysogens. The percent lysogeny was determined as follows: (number of lysogens/viable-cell count)  $\times$  100.

**(iv) Lysogen induction.** Induction of viable phage from a lysogen was quantified by a single-burst assay in which overnight-grown wild-type and *ssrA* lysogens were subcultured 1:50 and incubated for 2.5 h at 37°C, at which time an aliquot was removed from the culture (time = 0). Mitomycin C (Sigma) was added to a final concentration of 2  $\mu$ g/ml, and the cultures were returned to 37°C. Aliquots (1 ml) were removed at various times for the determination of phage production and viable cells. Phage production was determined by removing an aliquot, lysing the cells by vortexing them in chloroform, centrifuging them for 2 min, and transferring the supernatant to a fresh tube. Because phage P22-3657 is defective in tail protein production, P22 phage tails were added to each lysate in vitro by adding 6  $\mu$ l of P22 tail stock prepared as described previously (37), after which the lysates were incubated overnight at room temperature. PFU were determined by subjecting the lysates to titer determination on strain MST3357, which constitutively expresses P22 gene 9 tail protein.

**$\beta$ -Galactosidase assays.** To determine  $\beta$ -galactosidase levels, a single colony was inoculated into 1 ml of MES medium buffered to pH 5.5 and containing 0.05 mM Mg<sup>2+</sup> and was grown overnight at 37°C with shaking for 16 h.  $\beta$ -Galactosidase activities were determined by the method of Slauch and Silhavy (49). The activity of  $\beta$ -galactosidase is reported as 10<sup>3</sup> U per optical density at 600 nm unit per milliliter of cell suspension, where units are micromoles of *o*-nitrophenol (ONP) formed per minute ( $n = 3$  trials; standard deviations were  $\pm 10\%$  of the mean).

**DNA sequencing and analysis.** DNA was sequenced by the dideoxy chain termination method (46). Homology searches were conducted using the FASTA program (Wisconsin Package version 10.0; Genetics Computer Group, Madison,

TABLE 2. Mutations in *ssrA* confer a virulence defect in *S. enterica* serovar Typhimurium

Strain	Relevant genotype	CI $\pm$ SD ( <i>n</i> ) <sup>a</sup>
MT2055	$\Delta zff7503::K_n$ (17-kb deletion of <i>ssrA</i> region)	0.0044 $\pm$ 0.0009 (4)
MT2094	$\Delta zff7505::K_n$ (3.4-kb deletion of <i>ssrA</i> region)	0.0042 $\pm$ 0.0020 (3)
MT2121	<i>ssrA1::Tn10d-Tc</i>	0.0145 $\pm$ 0.0092 (4)
MT2194	$\Delta ssrA2::K_n$ deletion	0.0047 $\pm$ 0.0013 (5)
MT2122	<i>zff7508::Tn10d-Tc</i> (within <i>SlpA</i> )	1.62 $\pm$ 0.917 (3)
MT2123	<i>zff7509::Tn10d-Tc</i> (intergenic between <i>SlpA</i> and <i>Orf</i> )	1.04 $\pm$ 0.323 (5)
MT2124	<i>zff7510::Tn10d-Tc</i> (within <i>Orf</i> )	0.836 $\pm$ 0.234 (3)
MT2095	$\Delta zff7506::K_n$ (2.0-kb deletion including <i>HlyD</i> and <i>HlyB</i> )	0.760 $\pm$ 0.507 (3)
MT2096	$\Delta zff7507::K_n$ (3.0-kb deletion including <i>HlyB</i> and <i>AggA</i> )	1.57 $\pm$ 1.51 (3)

<sup>a</sup> Competitive index (CI) assays were performed by intraperitoneal infection of BALB/c mice with  $10^3$  total bacteria in a 1:1 ratio of mutant bacteria to wild type (MT2057). At 5 days after infection, bacterial cells were recovered from the spleen. The CI is the ratio of mutant to wild-type bacteria recovered. *n*, number of mice tested.

Wis.) and the BLASTN and BLASTX algorithms available at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

## RESULTS

***ssrA* is required for *Salmonella* virulence.** Previously, we have shown that a deletion of 17 kb of *Salmonella*-specific DNA at min 60 conferred a greater-than-200-fold virulence defect in BALB/c mice (10). To identify the gene(s) responsible, a series of defined deletions throughout the 17-kb region were constructed and tested for the ability to confer defects in virulence in a murine model for typhoid fever. Table 2 shows that removal of 3.4 kb of sequence (MT2094) conferred the same virulence defect as did the 17-kb deletion (MT2055). Sequence analysis of this region (Fig. 1) revealed three genes: *ssrA*, a predicted open reading frame with DNA homology (55%) to *E. coli* *slpA* integrase (27), and a predicted open reading frame of unknown function. *Tn10d-Tc* transposon insertions were isolated in each of these genes and retested for

virulence. Table 2 shows that *ssrA1::Tn10d-Tc* (MT2121) conferred a virulence defect (70-fold) whereas all other downstream insertion (MT2122 to MT2124) and upstream deletion (MT2095 and MT2096) strains exhibited wild-type virulence. Additionally, MT2194, which contains a deletion of sequences entirely within *ssrA*, reproduced the 200-fold defect, suggesting that *ssrA* was responsible for the virulence phenotype (Table 2).

***ssrA* is required for expression of *ivi* genes.** The effect of *ssrA* on *ivi* gene expression was determined by transducing an *ssrA* deletion ( $\Delta ssrA2::K_n$ ) into our collection of more than 200 *ivi* fusion strains (19). Figure 2 shows that *ssrA* affected the expression of six *ivi* genes, three of which (*vacB*, *asnS*, and *thiI*) were induced by *ssrA* and three of which (*f304*, *dedE*, and *yoaA*) were repressed by *ssrA*. Several of these genes are required for or implicated in virulence, including *vacB*, which is required for the expression of the invasion plasmid antigen proteins of *Shigella flexneri* (28); *f304*, an open reading frame of unknown function which resides within a uropathogenic *E. coli* pathogenicity island (16); and *dedE* (*cvpA*), which is required for the production of the colicin V toxin (14). Other genes under *ssrA* control include *asnS*, which encodes asparaginyl-tRNA synthase (57); *thiI*, which is involved in the production of thiazole, a precursor of thiamine synthesis (54); and *yoaA*, a homologue of the *dinG* family of DNA helicases involved in the bacterial SOS response (31).

***Salmonella*-specific sequence adjacent to *ssrA*.** Previously, we have shown that *ssrA* resides near the junction of two different regions of *Salmonella*-specific sequence (10). To ascertain whether *ssrA* was at the junction point of either of these unique DNA regions, the *ssrA* region was sequenced. *S. typhimurium* *ssrA* shows 96.1% homology to the *E. coli* *ssrA* gene. However, homology to *E. coli* is lost immediately after the 3' end of *ssrA*, which contains the P4-like bacteriophage attachment site, *attL* (27). *attL* serves as the attachment site for the *E. coli* cryptic prophage, CP4-57 (27), and pathogenicity islands in *Vibrio cholerae* (30) and *Dichelobacter nodosus* (2), the causative agents of cholera and sheep foot rot, respectively. These data suggest that *ssrA attL* sequences serve as the attachment site for one of the two regions of *Salmonella*-specific sequences at min 60. Moreover, *Salmonella*-specific sequence with limited

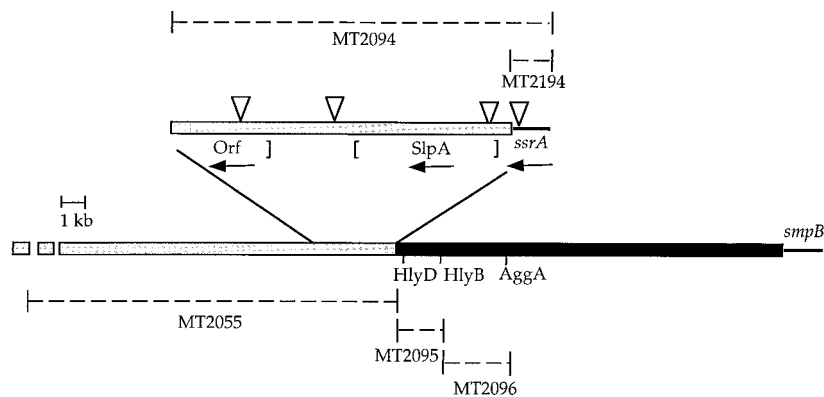


FIG. 1. Genetic organization of the *S. enterica* serovar Typhimurium *ssrA* region. Thick black and gray lines represent two distinct *Salmonella*-specific regions (10); thin black lines represent sequences shared between *S. enterica* serovar Typhimurium and *E. coli* (i.e., *ssrA* and *smpB*); dashed lines represent the extent of deleted DNA. The enlarged region denotes the material deleted in MT2094; *Tn10d-Tc* insertions in this region are designated by triangles; the arrows depict the predicted direction of transcription. *Salmonella*-specific sequences upstream of *ssrA* encode predicted proteins with similarity to sequences encoded within SPI4, an unlinked *S. enterica* serovar Typhimurium pathogenicity island (56). Protein similarities include *HlyD* and *LktD*, which are involved in the secretion of hemolysin in *E. coli* and *Pasteurella haemolytica*, respectively (32% identity over 176 amino acids and 26% identity over 146 amino acids to SPI4 protein D), ABC transport proteins (*HlyB* and *LktB* family, involved in the transport of hemolysin) (32% identity over 177 amino acids and 26% identity over 175 amino acids to SPI4 protein R), *AggA* and *PrtF*, which are involved in *Pseudomonas putida* adherence to root tip cells and protease secretion in *Erwinia chrysanthemi*, respectively (21% identity over 375 amino acids and 18% identical over 401 amino acids to SPI4 protein C).

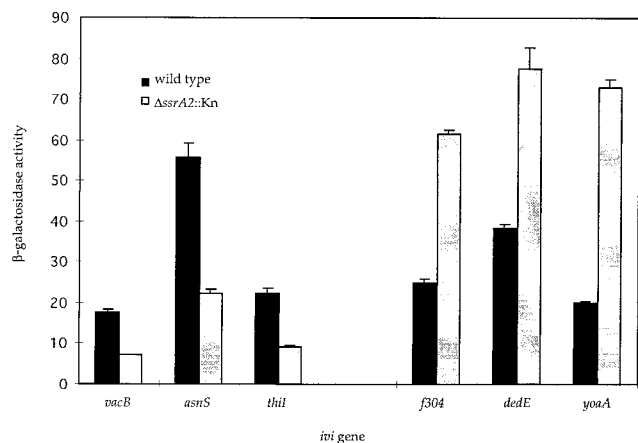


FIG. 2. *ssrA* regulation of *ivi* genes.  $\beta$ -Galactosidase activities were determined following overnight incubation in MES minimal medium buffered to pH 5.5 and containing 0.05 mM  $Mg^{2+}$ .  $\beta$ -Galactosidase activities are given as  $10^3$  micromoles of ONP formed per minute per optical density unit at 600 nm per milliliter of cell suspension. Values represent the average of three independent cultures, with standard deviations ( $\pm 10\%$ ) shown as error bars.

or no homology to *E. coli* continues for several kilobases 3' to the *ssrA attL* attachment site. The first 180 nucleotides downstream of *attL* have no homology to the GenEMBL DNA database, after which limited homology to the *E. coli* DNA sequence (55%) extends for 1.2 kb (Fig. 1). This coincides with the location of *E. coli slpA*, which encodes the cryptic phage CP4-57 integrase SlpA.

Analysis of the sequence at the 5' end of *ssrA* revealed homology to the *E. coli* sequence for 116 nucleotides (67%), after which no homology to the DNA database was detected for at least another 1.1 kb. No bacterial attachment sites or IS sequences were detected at or near the junction point of this region of *Salmonella*-specific sequence. Figure 1 shows that this region of *Salmonella*-specific sequence encodes predicted proteins with similarity to sequences encoded within SPI4, an unlinked *S. enterica* serovar Typhimurium pathogenicity island (56). Protein similarities include HlyD/LktD (SPI4 protein D), involved in the secretion of hemolysin in *E. coli* and *Pasteurella haemolytica*; HlyB/LktB family (SPI4 protein R), involved in the transport of hemolysin; and AggA/PrtF (SPI4 protein C), involved in *Pseudomonas putida* adherence to root tip cells and protease secretion in *Erwinia chrysanthemi*, respectively. Deletion of these SPI4-like sequences did not have an effect on virulence (Table 2).

***ssrA* is required for support of the growth of phage P22 and for the induction of P22 lysogens.** Table 3 shows that *S. enterica* serovar Typhimurium *ssrA* hosts are severely restricted (nearly 10,000-fold) in their EOP of phage P22. Moreover, the rare phage plaques produced on an *ssrA* host appeared to be mutant by two criteria: (i) the plaques produced on an *ssrA* host have a clear plaque morphology relative to the turbid plaques produced by phage P22 on wild-type *S. enterica* serovar Typhimurium (data not shown); and (ii) clear-plaque phage showed a wild-type EOP when plated on either *ssrA* or wild-type hosts (data not shown).

To characterize the EOP defect, we tested the effect of *ssrA* on phage P22 adsorption, P22 lysogen formation, and P22 lysogen induction. Table 3 shows that *ssrA* strains are proficient for phage adsorption, suggesting that the role of *ssrA* in supporting phage growth occurs at a postadsorption step. The efficiency of P22 lysogeny of *ssrA* strains was tested using the kanamycin-resistant phage P22-3657 (15), which allows direct

selection for phage P22 lysogens. Table 3 shows that P22-3657 was able to lysogenize an *ssrA* strain (MT2121) with wild-type efficiency, indicating that the lack of *ssrA* did not impair the formation of stable phage P22 lysogens.

The ability to produce viable phage from P22 lysogens was tested in *ssrA* strains. Phage P22-3657 was also used in this analysis since it lacks the P22 tail protein (encoded by gene 9 [15]), and thus progeny phage are unable to reinfect other host cells. Single-step phage production from P22-3657 lysogens was assessed in wild-type and *ssrA* backgrounds. Following lysogen induction with mitomycin C, bacterial cells were lysed with chloroform at several time points to quantitate the phage production. Figure 3 shows that the *ssrA1::Tn10dTc* lysogen exhibited a 40-min delay in the production of phage following the addition of mitomycin C. However, despite the delay, the titer of viable phage in *ssrA* lysogens after 3 h of induction was indistinguishable from the titer obtained in wild-type host strains. The phage growth delay was not due to differences in host strain survival in response to phage induction, since the wild-type and *ssrA* lysogens displayed very similar viable-cell counts over time in response to mitomycin C treatment (data not shown). Moreover, the 40-min delay in recovery of viable phage was not due to a phage P22 lysis defect, since the host cells were lysed before viable phage were quantified. Taken together, these data suggest that *ssrA* is required for lytic growth of phage P22 and for the induction of a P22 lysogen in *S. enterica* serovar Typhimurium.

## DISCUSSION

*ssrA* encodes a multifunctional RNA molecule that plays many diverse roles in the bacterial cell, ranging from tagging abnormal proteins for degradation to supporting phage growth to modulating the activity of DNA binding proteins. Here we show that *ssrA* (i) is required for full virulence in *S. enterica* serovar Typhimurium, (ii) affects the expression of several genes induced during infection, (iii) is the integration site for a

TABLE 3. Effects of *S. enterica* serovar Typhimurium *ssrA* on EOP, phage P22 adsorption, and formation of P22 lysogens

Strain	Relevant genotype	EOP <sup>a</sup>	% P22 adsorption <sup>b</sup> (P22 HT 105/1, <i>int</i> -201)	% P22 lysogeny <sup>c</sup> (P22-3657)
14028	Wild type	1	99.5 $\pm$ 0.17	41.1 $\pm$ 4.1
MT2121	<i>ssrA1::Tn10d-Tc</i>	1.11 $\times 10^{-4}$	99.8 $\pm$ 0.06	40.9 $\pm$ 5.3
MT2194	$\Delta ssrA2::Kn$	1.05 $\times 10^{-4}$	99.7 $\pm$ 0.06	ND <sup>e</sup>
DH5 $\alpha$ $\lambda$ pir	<i>E. coli</i>	NA <sup>d</sup>	4.6 $\pm$ 0.70	ND

<sup>a</sup> EOP experiments were performed by plaquing P22 HT 105/1, *int*-201 on the recipient strain indicated. The EOP of the wild-type strain was defined as 1; the EOP of the mutant strains was calculated as follows: (number of PFU/ml obtained on the mutant strain)/(number of PFU/ml obtained on the wild-type strain). Values represent the average of three independent experiments with a standard deviation of <10% of the mean.

<sup>b</sup> P22 adsorption assays were performed by incubating the generalized transducing phage mutant P22 HT105/1, *int*-201 with bacteria for 15 min at 37°C, at which time the cells were centrifuged and the supernatants were recovered. Values are reported as the percentage of phage recovered, according to the formula [(PFU of phage without cells) - (PFU of phage with cells)]/PFU of phage without cells  $\times 100$ . Values represent the average of three independent experiments  $\pm$  standard deviation.

<sup>c</sup> P22 lysogeny experiments were performed using phage P22-3657, which contains a kanamycin resistance gene in place of the phage *mnt* gene (15), allowing direct selection of lysogens. The percent lysogeny was calculated as follows: (number of lysogens/viable-cell count determined at the time when cells and phage were mixed)  $\times 100$ . Values represent the average of three independent experiments  $\pm$  standard deviation.

<sup>d</sup> NA, not applicable.

<sup>e</sup> ND, not determined.

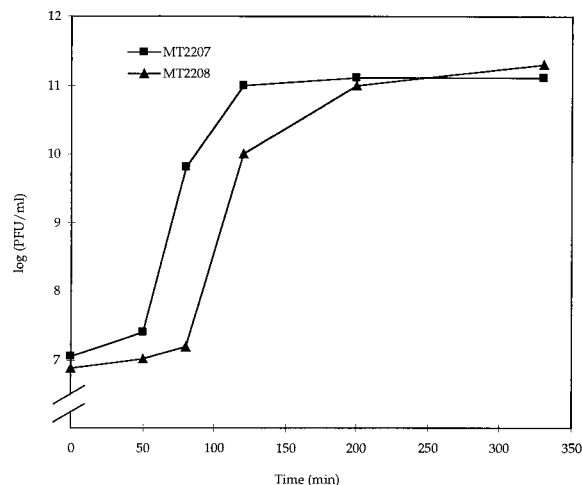


FIG. 3. Effect of *ssrA* on the induction of phage P22 lysogens. Phage production in wild-type (MT2207) and *ssrA* (MT2208) strains [represented as the log (PFU/ml) on the y axis] is shown as a function of time after mitomycin C induction of a P22-3657 lysogen. P22-3657 contains a deletion of P22 tail gene 9, and thus single-burst progeny phage in the supernatant were subjected to titer determination on strain MST3375, which constitutively expresses the P22 tail protein. Values represent the average of three independent experiments, where the standard deviation is <20%.

large region of *Salmonella*-specific sequence, and (iv) is required to support the growth of bacteriophage P22 and for the induction of P22 lysogens.

The role of *ssrA* in *Salmonella* virulence may be attributed to its known role as a tmRNA that tags aberrant polypeptides for degradation. Keiler et al. (26) proposed that tmRNA initially functions as a tRNA and then as an mRNA, resulting in the release of the parental mRNA template from the ribosome and translocation of the nascent peptide to tmRNA. The subsequent addition of an tmRNA-encoded tag marks the nascent polypeptide for degradation (26). In the macrophage phagosome, reactive oxidants are a major source of DNA and/or protein damage and play a major role in bacterial killing (1). Accordingly, the repair of damaged DNA/or protein has been implicated in *Salmonella* pathogenesis (3). *ssrA* may counter, in part, the oxidative damage by preventing the buildup of abnormal proteins which may be toxic to the cell.

Another role of *ssrA* in *Salmonella* pathogenesis may be its effect on the expression of virulence functions since RNA molecules are known to control protein production at the transcriptional (e.g., transcriptional silencing [50]) and posttranscriptional (e.g., antisense [12, 22]), anti-antisense [36], and mRNA decay [32, 43] mechanisms) levels. Moreover, small RNA molecules which control the expression of virulence genes and functions have been identified for several pathogens including (i) tRNA molecules implicated for translation of virulence genes within pathogenicity islands of uropathogenic *E. coli* (17, 42) and type 1 fimbriae in *Salmonella* spp. (9, 52); (ii) RNAIII, a global *Staphylococcus aureus* regulator involved in the expression of several extracellular toxins and cell surface proteins (39); and a putative *Erwinia carotovora* RNA molecule, AepH, an activator of extracellular proteases involved in tissue maceration in plant hosts (38).

Similarly, a subset of the *ssrA*-regulated genes identified in this work are required for or implicated in virulence: *vacB* is an RNase R which regulates the production of proteins required for invasion, intracellular dissemination, and macrophage apoptosis in *Shigella flexneri* (6, 28); *f304* is contained within a pathogenicity island in uropathogenic *E. coli* strain CFT073,

and its presence has been correlated with more acute clinical symptoms (16, 24); *dedE* is required for the production of the toxin colicin V (14); and *yoaA* encodes a homologue of the *dinG* family of DNA helicases involved in the bacterial SOS response (31). Additionally, *ssrA* may affect the expression of other virulence genes in *Salmonella*.

The integration sites for pathogenicity islands are often within genes encoding small RNA molecules, e.g., tRNA genes (17). Similarly, a portion of the bacteriophage P4 attachment site, *attL*, resides within the 3' end of *ssrA*, which serves as the insertion site for acquired sequences in several organisms, including the cryptic phage CP4-57 in *E. coli* (27), and pathogenicity islands in *Vibrio cholerae* (25, 30) and *Dichelobacter nodosus* (2). Here we show that the *ssrA attL* is also the attachment site for a region of acquired sequence in *Salmonella*. Additionally, another region of acquired sequence maps 5' to *ssrA*, integrating between the corresponding *ssrA* and *smgB* genes in *E. coli*. Sequence analysis of the *ssrA*-proximal junction region did not reveal bacteriophage attachment sites, IS sequences, or sequence with significant repeats, and thus the mechanism of inheritance of this region remains unclear.

The temperate bacteriophage P22 is subject to complex regulatory mechanisms (51). Here we have shown that *Salmonella ssrA* mutants were deficient in the ability to support the growth of phage P22, as evidenced by a severe reduction in the EOP (nearly 10,000-fold). *ssrA* mutants also showed a delay in the production of viable P22 phage when induced from a lysogen, which may arise from inefficient phage P22 excision or from an inability to effectively initiate and/or sustain phage replication. Retallack et al. (40) have shown that *ssrA* mutants impair the growth of hybrid  $\lambda$ /P22 phage in an *E. coli* host. However, phage mutants that lack the C1 gene product, which activates transcription of the P22 repressor (*c2*), produce phage at wild-type levels in *E. coli ssrA* hosts. These researchers propose a model in which tmRNA directly or indirectly affects the expression of C1-controlled genes/functions required for phage growth. Their model suggests that the lack of tmRNA alters the affinity of a phage-regulatory protein(s) for its cognate DNA binding site (40) and/or leads to a deficiency of aminoacylated tmRNA molecules required for translation of phage mRNA (55). In addition to the defects in phage P22 growth presented here for *S. enterica* serovar Typhimurium, tmRNA-mediated alterations in DNA-protein interactions and/or translation may contribute to the role of *ssrA* in bacterial gene expression and in the virulence of *Salmonella* and other pathogens.

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