A Novel sRNA in Shigella flexneri That Regulates Tolerance and Virulence Under Hyperosmotic Pressure

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Regulation of the environmental stress response and virulence of Shigella flexneri may involve multiple signaling pathways; however, these mechanisms are not well-defined. In bacteria, small regulatory RNAs (sRNAs) regulate bacterial growth, metabolism, virulence, and environmental stress response. Therefore, identifying novel functional sRNAs in S. flexneri could help elucidate pathogenic adaptations to host micro-environmental stresses and associated virulence. The aim of this study was to confirm the presence of an sRNA, Ssr54, in S. flexneri and to determine its functions and possible mechanism of action. Ssr54 was found to regulate tolerance and virulence under hyperosmotic pressure. Its expression was verified by qRT-PCR and Northern blotting, and its genomic position was confirmed by 5′-rapid amplification of cDNA ends. Ssr54 expression was significantly decreased (~80%) under hyperosmotic conditions (680 mM NaCl), and the survival rate of the Ssr54 deletion strain increased by 20% under these conditions. This suggested that Ssr54 has been selected to promote host survival under hyperosmotic conditions. Additionally, virulence assessment, including guinea pig Sereny test and competitive invasion assays in mouse lungs, revealed that Ssr54 deletion significantly decreased S. flexneri virulence. Two-dimensional gel analyses suggest that Ssr54 may modulate the expression of tolC, ompA, and treF genes, which may affect the virulence and survival of S. flexneri under osmotic pressures. Furthermore, treF expression has been shown to improve the survival of S. flexneri under osmotic pressures. These results suggest that Ssr54 has a broad range of action in S. flexneri response to hyperosmotic environmental stresses and in controlling its virulence to adapt to environmental stresses encountered during host infection.

Keywords: Shigella flexneri, sRNA, environmental stress, virulence, Ssr54
INTRODUCTION

Shigella is a genus of gram-negative enteric bacteria, accounting for some of the most prominent pathogens causing bacterial diarrhea via fecal-oral route. Shigella infect colonic epithelial cells, leading to typical bacterial diarrheal symptoms. S. flexneri is the most prevalent endemic strain in developing countries (Kotloff et al., 1999; Wang et al., 2006). When S. flexneri enters the host body, it faces various host gastrointestinal environmental stresses characterized by fluctuating temperature, nutrients, bile salts, and pH conditions. Adaptations to these environmental stresses are important for S. flexneri survival and subsequent invasion and colonization of the intestinal tract, which are responsible for the pathogenic effects of the infection. To adapt, the bacteria require coordinated regulation of gene expression for rapid responses to and tolerance of these stresses. For instance, in S. flexneri, acid resistance is dependent on pH and controlled by RpoS under stress conditions (Small et al., 1994). Meanwhile, the two efflux pumps, AcrAB and MdtJI, of Shigella are reportedly associated with bile salts and the extrusion of toxic compounds (Leuzzi et al., 2015; Nickerson et al., 2017). Hence, developing strategies for tolerating changes in environmental osmolarity, is critical for pathogenic bacteria to not only permit survival during environmental osmotic stress, but also to establish infection (Mahmoud et al., 2017). Specifically, maintaining a stable osmotic balance between the cell cytoplasm and outer environment is an important challenge for all cell types, particularly bacteria.

Different bacteria respond to environmental stresses via different strategies, including the use of small regulatory RNAs (sRNAs). Most sRNAs are located in intergenic regions that range from 40 to 500 nt. They affect mRNA activity by base pairing with the 5′-untranslated regions (5′-UTRs) and/or the ribosome binding site (RBS) of these targets (Gottesman and Storz, 2011; Vanderpool et al., 2011). Antisense sRNAs, transcribed on the DNA strand opposite their target gene, are one type of sRNAs that act via extensive base pairing (Wagner and Romby, 2015). sRNAs have been identified in many bacteria, including Escherichia coli, Staphylococcus aureus, and Vibrio cholerae, in which they play pivotal roles in bacterial adaptation to environmental stress and virulence (Lenz et al., 2004; Mangold et al., 2004; Pichon and Felden, 2005; Bobrovskyy and Vanderpool, 2013; De Lay et al., 2013; Murina and Nikulin, 2015). For example, E. coli DsrA can bind to RpoS mRNA, which encodes the alternative sigma factor S, to repress formation of a closed secondary structure in the RBS of rpoS transcripts and promote translation (Massé and Gottesman, 2002). Moreover, E. coli sRNA RyhB regulates intracellular Fe^{2+} utilization and storage, as well as iron binding proteins (Massé et al., 2005). sRNA also affects the pathogenesis of gram-positive Enterococcus faecalis by regulating its growth and survival under various environmental stresses, including iron stress (Michaux et al., 2014). In fact, studies have suggested that 10–30% of all bacterial genes are subjected to sRNA regulation (Lewis et al., 2005).

Various studies have also reported on sRNA function in Shigella. For instance, in S. dysenteriae, the sRNA RyfA1 negatively impacts the virulence-associated process of cell-to-cell transfer, as well as expression of ompC, a gene that is important for the pathogenesis of Shigella (Fris et al., 2017). Additionally, CsrB/CsrC is regulated in response to carbon availability, RyhB is regulated in response to iron availability, while RnaG, as well as the activity of the shuA RNA thermometer, is regulated in response to temperature (Fris and Murphy, 2016). RnaG (450 nt), a small non-coding RNA encoded on the virulence plasmid pINV of Shigella flexneri, is able to directly interact with icsA mRNA, thereby promoting premature termination of transcription (Giangrossi et al., 2010) Additionally, VirF, which generally assists the RNA–RNA interaction, has been shown to directly activate the transcription of the small non-coding RnaG and icsA mRNA, which encodes two essential proteins that are involved in the pathogenicity process by binding their promoter regions (Giangrossi et al., 2017). RyhB (90 nt) modulates and inactivates ferritin-related genes to regulate iron metabolism and can inhibit the expression of virulence genes such as virB, a regulator of the T3SS secretory protein gene (Murphy and Payne, 2007). Meanwhile, in our previous study, Sorl (150 nt) was shown to help bacteria adapt to an acidic environment by regulating acid resistance genes and to regulate bacterial virulence by controlling the expression of T3SS genes (Wang L. et al., 2016). However, the functions of many other predicted sRNAs in Shigella have not yet been elucidated. The identification of novel S. flexneri sRNAs is pivotal to understanding Shigella virulence and adaptation to host microenvironment. Therefore, the aim of the present study was to confirm the presence of an sRNA, Ssr54, in S. flexneri that was predicted using a comparative genomics bioinformatics screening method (Wang L. et al., 2016), as well as determine the functions and possible mechanisms of action of this sRNA in S. flexneri.

MATERIALS AND METHODS

Bacterial Strains, Growth Media, and Stress Conditions

The wild-type strain used in this study was S. flexneri 2a 301. This strain was cultured on Congo red solid culture medium at 37°C in 5% CO_{2} overnight. A single colony was selected, inoculated in liquid Luria-Bertani (LB) medium, and incubated at 37°C on an orbital shaker at 150 rpm for 6–8 h. When necessary, kanamycin, ampicillin, or chloramphenicol was added to the culture medium to obtain a final concentration of 50, 100, or 30 µg/mL, respectively. In the stress test, a bacterial culture with an OD_{600} of 0.6 was pelleted, resuspended in PBS, and inoculated into the following culture media: a hypoosmotic LB culture medium not containing NaCl, hyperosmotic LB culture media containing 340 or 680 mM NaCl, and LB culture medium with 160 mM NaCl as the negative control. The bacteria were cultured under these conditions for 30 min. Thereafter, we assessed sRNA expression by qRT-PCR and performed the survival assay under hyperosmotic conditions.

For the northern blot analysis, wild-type, Ssr54 mutant, and Ssr54 complement strain cells were harvested during the logarithmic growth phase (OD_{600} = 0.6). When required, the cultures were subjected to hypo/hyperosmotic stress conditions.
as described above. Non-stressed exponentially growing cultures were used as controls.

**RNA Extraction and RT-PCR**

Total RNA was isolated from S. flexneri cultured to the middle logarithmic growth phase (OD$_{600}$ = 0.6), and remaining DNA was digested with DNase (Promega, Madison, WI, USA, M6101). ODs were determined at 260 nm and 280 nm using a UV-Vis spectrophotometer to assess RNA concentration. DNase-treated total RNA (not more than 10 µg) was reverse-transcribed into cDNA using a reverse transcription kit (Promega, Madison, WI, USA). cDNA was synthesized using 2 µg of total RNA and a Promega Reverse Transcription System. It was then diluted (1:5) in nuclease-free water. Primers were designed based on the Ssr54 sequence (Supplementary Table 1 for further details) and used the cDNA as PCR template. PCR was performed using Ex Taq Mix (Takara Biochemicals, Beijing, China, RR001Q) under the following thermal cycling conditions: 10 min at 95°C for preincubation, followed by 30 cycles of amplification (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C) (Absin Bioscience, Foster City, CA, USA). RNA preparation and qRT-PCR analysis were repeated at least three times.

**Northern Blot**

Ssr54 size and expression levels were assessed by northern blotting with total RNA from the wild-type, Ssr54 mutant, and complement strains in normal and hyperosmotic conditions. Total RNA (15–20 µg from each strain) was separated via denaturing polyacrylamide gel electrophoresis (PAGE) (10% resolving gel) and electroblotted onto a nylon membrane where it was cross-linked under a 1,200-mJ UV light. The Ssr54 probe sequence was 5'-ATTCTTTCAGAGATCACAACACTGTTTA-3'. The sequence of 5S rRNA probe, which was used as the internal control, was 5'-GTTTCACTCTTGCTGCGATGCTGTTGTTGGG-3'. These probes were synthesized by Shanghai Invitrogen Life Technology Service (Shanghai, China). The RNA was then hybridized with a biotin-labeled Ssr54 probe overnight, and the hybridization signal was detected using a North2South Chemiluminescent Hybridization and Detection Kit from Thermo Fisher Scientific (Carlsbad, CA, USA, 17097) with an exposure time of 1–5 min.

**Mapping of sRNA by Rapid Amplification of cDNA Ends (RACE)**

The precise size of Ssr54 was determined by RACE using the 5'-Full RACE Kit (Takara Biochemicals, Beijing, China, D315) in accordance with the manufacturer's instructions. Total RNA was isolated from S. flexneri 2a 301 during the exponential phase (OD = 0.6). The Ssr54 RACE outer primer and inner primer sequences are presented in Supplementary Table 1. The resulting purified sRNA-derived PCR product was cloned into the pMD18T vector and transformed into competent E. coli DH5α cells. The inserts were sequenced to identify the TSSs of the individual sRNAs.

**qRT-PCR**

qRT-PCR was performed to assess Ssr54 sRNA expression in S. flexneri under different osmotic pressures. cDNA was synthesized using 2 µg of total RNA and a Promega Reverse Transcription System and then diluted (1:5) in nuclease-free water. With 3 µL of cDNA as the template and 16S rRNA as the internal control, Ssr54 was amplified by PCR using the 2x SYBR Green I Master Mix protocol (Invitrogen, Carlsbad, CA, USA). The primers are presented in Supplementary Table 1. RT-PCR was performed using the ABI 7500 system (Applied Biosystems) with the following cycling conditions: 5 min denaturation step at 42°C, followed by 40 amplification cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Ssr54 gene transcript levels under various stress conditions were compared with those of the wild-type strain grown in normal LB medium at 37°C. Relative expression levels were calculated using the 2$^{-\Delta\Delta CT}$ method (Gong et al., 2011). All experiments were conducted in triplicate.

**Construction of sRNA Mutant and Complement Strains**

The Ssr54 mutant strains were generated through λ-Red homologous recombination (Park et al., 2001). Genome segments 500–600 bp upstream and downstream of the homologous arm fragments in the Ssr54 sRNA sequence were amplified. The kanamycin resistance gene from the pUC19-kan plasmid was amplified, and the fusion-PCR method (with three fragments at a ratio of 1:1:1) was employed to generate a linear target DNA fragment. This linear fragment was transformed into a S. flexneri strain containing the pKD46 plasmid, which encoded the λ-Red recombination system, and the strain was cultured overnight on an antibiotic selection plate containing ampicillin and kanamycin to screen for the Ssr54-deficient mutant strain. This strain was confirmed using primers listed in Supplementary Table 1.

To construct the complementing plasmid pSsr54, DNA 200 bp upstream of the Ssr54, transcription start site (TSS), and its coding region was amplified from S. flexneri 2a 301. These primers targeted unique restriction enzyme sites, XbaI and Sphi, such that when the PCR product was digested with these restriction enzymes, it could be ligated into a similarly digested plasmid, recombinant pACYC184 (CmR, TcR3), in an orientation-specific manner. Recombinant DNA products were verified by sequencing. The resulting plasmid was used to transform the Ssr54 mutant strain via electroporation, followed by selection using chloramphenicol. The final complement strain was dubbed ΔSsr54 + pSsr54, pACYC184 was electroporated into these strains to generate vector-only (no sRNA) controls.

**Growth Analysis of S. flexneri**

Overnight cultures grown in LB medium or LB medium containing kanamycin were diluted 1:100 in LB medium with the hyperosmotic supplement and then grown at 37°C with shaking at 200 rpm/min. At time points of 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h after inoculation, bacterial growth was monitored by measuring the OD$_{600}$. Each sample was analyzed in triplicate and the assays were repeated at least three times. The average OD$_{600}$ values were used to generate growth curves.
**Determination of Survival Rates**

The wild-type and Ssr54 mutant strains were cultured at 37°C with agitation at 160 rpm until the early logarithmic phase of growth (OD\text{600} of 0.6). Bacterial cultures were centrifuged for 5 min at 4°C; then, pellets were washed three times in PBS, resuspended in hypertonic LB culture medium containing 680 mM NaCl, and cultured for 30 min at 37°C (Gong et al., 2011). Final bacterial cultures were serially diluted and plated onto LB plates for overnight incubation to determine viability. The number of colony-forming units per milliliter (CFU/mL) was then calculated for each of the conditions, with each bacterial strain cultured in LB culture medium at pH 7.0 (containing 160 mM NaCl) serving as the control. Results are expressed as mean percentage ± SD from experiments performed in triplicate.

**Induction of Keratoconjunctivitis in Guinea Pigs**

Analysis of keratoconjunctivitis induced in guinea pigs is a standard method to assess *S. flexneri* virulence, and it allows for direct evaluation of *S. flexneri* pathogenesis (Sereny, 1955). To verify the effect of Ssr54 sRNA on *S. flexneri* virulence, we investigated the effect of the wild-type, Ssr54 mutant, and complement strains on the corneas of guinea pigs. Single colonies of the wild-type, Ssr54 mutant, and Ssr54 complement strains were selected from TSA Congo red plates and inoculated in LB culture medium containing the appropriate antibiotics for incubation at 37°C overnight. Thereafter, 1% of each culture was inoculated into 5 mL of LB culture medium and incubated until it reached an OD\text{600} of 1.0 (10\textsuperscript{6} CFU/mL). After centrifugation for 5 min, we harvested 1 mL of each bacterial culture and resuspended it in 20 µL of PBS, which was then inoculated into the corneas of healthy guinea pigs with normal cornea and conjunctiva. Inoculated corneas were observed 24, 48, and 72 h after inoculation. Corneas inoculated with PBS served as controls. The degree of keratoconjunctivitis was graded as follows: – no keratoconjunctivitis; + mild conjunctival congestion; ++ palpebral edema, conjunctival congestion, corneal opacification, and no purulent discharge; +++ palpebral edema, corneal opacification, conjunctival congestion, and purulent discharge; +++++ palpebral edema, corneal gray patch, conjunctival congestion, and purulent discharge. The experiment was conducted in triplicate. Each group contained six guinea pigs.

**Assessment of Mouse Lung Invasion**

The wild-type, mutant Ssr54, and complement strains were grown to the logarithmic growth phase, and then the concentrations of cultures were adjusted to 10\textsuperscript{8} CFU/mL. Three aliquots (1 mL each) containing the same number of bacteria were pelleted by centrifugation for 5 min at 4°C and resuspended in 1 mL of PBS. Equal amounts of the wild-type and mutant strains were mixed, and an aliquot (20 µL) of the resulting mixture (2 × 10\textsuperscript{6} CFU/mL), along with a similar aliquot of the mixture of equal amounts of wild-type and complement strains, were placed in the nasal cavities of BALB/c mice, under diethyl ether sedation, to infect lung tissues. After 24 h, the mice were euthanized via cervical dislocation, and lungs were collected from all and washed in RPMI (Roswell Park Memorial Institute) 1640 with 50 µg/mL gentamicin. Mouse lungs were harvested and incubated in PBS containing 0.1% Triton X-100 to release the bacteria. The extracts were then spread on either antibiotic-free or kanamycin antibiotic-containing LB plates for overnight cultivation at 37°C, and then the numbers of colonies were determined. The competitive index (CI) was defined as the ratio of mutant strain CFU after invasion to that of the wild-type strain CFU after invasion, divided by the ratio of the mutant strain CFU before invasion to that of the wild-type strain CFU before invasion. This experiment was performed in triplicate.

**HeLa Cell Invasion Assays**

To further verify the influence of Ssr54 on virulence in *S. flexneri*, a competitive invasion assay was performed using HeLa cells. *S. flexneri* strain 301, Ssr54 mutant and complement strains were grown in LB medium to the exponential phase (OD\text{600} of 0.6), adjusted to the same concentration, and mixed at a 1:1 (v/v) ratio. The two strains were added to DMEM cell culture medium (Gibco, Carlsbad, CA, USA), and the cultures formed were then added to HeLa cells at a multiplicity of infection of 100:1. After incubation for 4 h, infected HeLa cells were washed thrice with PBS and thrice with cell culture medium, following which cell culture medium containing 50 µg/mL gentamicin was added (Wang Y. et al., 2011). Gentamicin kills extracellular bacteria, while intracellular bacteria are protected from it. Following incubation at 37°C for 24 h, cell cultures were washed with PBS; the cultures were then harvested and lysed, and their cellular contents were cultured on LB and LB plates containing kanamycin. The CI was calculated, which represents the ratio of the CFUs of the recovered Ssr54 mutant and the wild-type, complement strains and the wild-type (normalized for the input ratio), and Student’s t-test was used to determine the p-value (Christiansen et al., 2004). This assay was repeated three times, and the mean was reported as the final result.

**Prediction of sRNA Targets**

Proteins from the wild-type and Ssr54 mutant strains were extracted and purified. The protein samples (total of 120 µg, as assessed by silver staining) were subjected to one-dimensional isoelectric focusing using IPG immobile dry strip gels at pH 4–7 for 30 min at 300 V, and then 30 min at 700 V, 1.5 h at 1,500 V, and 8 h at 9,000 V until the voltage-time product reached 64 kVh. Thereafter, isoelectric focusing was discontinued, and the samples were separated in the other direction by sodium dodecyl sulfate (SDS)-PAGE (12.5% resolving gel). The gels were then stained, scanned, and subjected to image analysis. The samples underwent protein mass spectroscopy pre-treatment processes, including determination of restriction enzyme digestion sites, enzymatic digestion, peptide extraction, identification of target proteins, and mass spectroscopy measurement.

**Ethics Statement**

The animals were obtained from the Laboratory Animal Center (Academy of Military Medical Sciences). All methods were...
carried out in accordance with the approved guidelines of the Academy of Military Medical Sciences. The experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Academy of Military Medical Sciences.

**Biosecurity Statement**

All standard biosecurity and institutional safety procedures were adhered to in all experimental procedures presented in this article.

**RESULTS**

**Identification of a Novel sRNA**

In our previous study, we predicted 57 sRNAs using a transcription unit-based method and confirmed four of these sRNAs (Ssr1, Ssr9, Ssr44, and Ssr54) by reverse transcription (RT)-PCR and northern blotting assays (Wang L. et al., 2016). In the current study, we focused on determining the characteristics and functions of Ssr54. sRNA Ssr54 expression was detected by RT-PCR and verified by northern blotting. A biotin-labeled Ssr54 probe was hybridized with a band of ~100 bp in a wild-type *S. flexneri* strain and
**Ssr54** complement strain; no band was seen in the **Ssr54** deletion strain (**Figure 1A**). In this experiment, 5S rRNA was used as the positive control. By 5′-RACE, the **Ssr54** TSS was at 3635111 and located in a non-coding intergenic region (**Figure 1B**).

**Ssr54 Regulates Osmolarity Tolerance**

We then examined whether **Ssr54** is required for *S. flexneri* to cope with osmotic stress. To this end, we first quantified its expression in osmotic conditions. Northern blot analysis was used to examine the levels of **Ssr54** sRNA in *S. flexneri* grown under various hyperosmotic conditions supplemented with 340 mM and 680 mM NaCl (**Figure 2A**). At osmotic pressures of 340 mM NaCl and 680 mM NaCl, the expression of **Ssr54** was predominantly low in the wild-type strain (**Figure 2A**). In this experiment, 5S rRNA was used as the positive control.

Reverse transcription quantitative PCR (RT-qPCR) was performed to further assess **Ssr54** expression in *S. flexneri*...
under different osmotic pressures. *Ssr54* was significantly downregulated under hyperosmotic pressures of 680 mM NaCl (>90% downregulation, *P* = 0.0231) and 340 mM NaCl (>80% downregulation, *P* = 0.0102) (Figure 2B). These results were consistent with the northern blot results, confirming changes in *Ssr54* expression under different osmotic pressures.

To investigate the role of *Ssr54* in adaptation of *S. flexneri* to hyperosmotic pressures, we constructed an *Ssr54* mutant strain using λ-red homologous recombination and a low-copy plasmid *Ssr54* complement strain. All mutant and complementary *Ssr54* constructs were confirmed by PCR and agarose gel electrophoresis (Supplementary Figure 1). To determine whether *Ssr54* plays a role in regulating growth under hyperosmotic conditions, we placed the wild-type, *Ssr54* mutant, and complement strains in LB culture medium under different stress conditions at 37°C for 24 h. Optical densities at 600 nm (OD$_{600}$) were measured every 2 h until the late stationary phase (24 h), and growth curves were plotted. A mutant carrying an empty vector served as a negative control. Under hypotonic conditions (0 mM NaCl), growth of the wild-type strain was not significantly different from that of the *Ssr54* mutant and complement strains (Figure 3A). Under hyperosmotic conditions (680 mM NaCl), the OD$_{600}$ values for the *Ssr54* mutant strain were significantly higher than those for the wild-type and *Ssr54* complement strains, and the *Ssr54* mutant strain entered the stationary phase later than the wild-type, hence indicating that entry into the stationary phase was accelerated in the deletion strain (Figure 3B). This suggested that *Ssr54* expression plays an important role in *S. flexneri* growth under hyperosmotic conditions.

In addition, we assessed survival of the wild-type and *Ssr54* mutant strains for 30 min following hyperosmotic stimulation and found no significant difference in growth. This survival assay was used to verify survival of the wild-type and *Ssr54* mutant strains under different osmolarities. After exposure to hyperosmotic pressure, survival of the *Ssr54* mutant strain improved by ~20% compared with that of the wild-type strain (*P* = 0.0109) (Figure 4). There was no difference in survival between the *Ssr54* complement strain and wild-type strain with the empty plasmid (*P* = 0.0701), indicating *Ssr54* deletion enhanced *S. flexneri* tolerance to hyperosmotic pressure and survival. The empty vector mutant served as a negative control.
Ssr54 Regulates Virulence

The wild-type, Ssr54 mutant, and complement strains were inoculated into the corneas of healthy guinea pigs (Figure 5). After 24h of infection, mild conjunctival congestion was observed in the wild-type and complement strain-inoculated group, whereas no keratoconjunctivitis was detected in the Ssr54 mutant strain-inoculated group or the PBS (control) group. After 48h of infection, the wild-type and complement strain-inoculated group presented palpebral edema and corneal opacification, and keratoconjunctivitis was still absent in the Ssr54 mutant strain-inoculated and PBS (control) groups. After 72h of infection, purulent discharge was observed in the wild-type and complement strain-inoculated group, and keratoconjunctivitis still remained absent in the Ssr54 mutant strain-inoculated and PBS (control) groups (Table 1). These data indicate that there was significant inflammatory cell infiltration.
following inoculation with the wild-type strain; however, there was no inflammatory response following inoculation with the Ssr54 mutant strain or PBS (Supplementary Figure 3). These data, which show the induction of keratoconjunctivitis in guinea pigs, strongly suggest that the inflammatory response time and infectivity of the Ssr54 mutant strain were significantly less than those of the wild-type and complement strains and imply that deletion of Ssr54 attenuates S. flexneri invasiveness.

Virulence of the wild-type, Ssr54 mutant, and complement strains was also assessed using a competitive invasion model in mouse lungs. Following competitive lung invasion by the wild-type, Ssr54 mutant, and complement strains, total viability of the Ssr54 mutant strain (2.07 \times 10^7) (6 replicates) was found to be significantly lower than that of the wild-type strain (6.15 \times 10^8) (P = 0.0068), whereas total viability of the complement strain (5.86 \times 10^4) (P > 0.05) was not significantly different from that of the wild-type strain. The CI between the Ssr54 mutant and wild-type strains was 0.028 (P < 0.05), which indicates a significant difference, whereas that between the Ssr54 complement and wild-type strains was 0.958 (P > 0.05), which was not significantly different (Figure 6). This finding suggested that deletion of Ssr54 significantly attenuated S. flexneri virulence, which was consistent with the experimental data regarding keratoconjunctivitis induction in guinea pigs.

In addition, we assessed the virulence of the wild-type, Ssr54 mutant, and complement strains in an in vivo HeLa cell invasion assay. The CFU recovered for the S. flexneri wild-type strain had a mean value of 1.696 \times 10^7 (6 replicates); the mean CFU of the wild-type was significantly higher than that of the Ssr54 mutant (1.3 \times 10^6 CFU (6 replicates); P < 0.05). The calculated median value for the CI values between the Ssr54 mutant and wild-type strains was 0.0865 (Figure 7). This value being significantly <1 (Student’s t-test, P < 0.05) strongly suggests that Ssr54 deletion has an impact on the ability of S. flexneri to effectively invade epithelial cell in vivo. However, the total viability of the complement strain (1.7 \times 10^7) (6 replicates) was not significantly different from that of the wild-type strain (P > 0.05). The CI between the Ssr54 complement and wild-type strains was 1.086 (P > 0.05), which was not significant (Figure 7).

### Determination of Ssr54 Targets

To determine the targets of Ssr54, whole bacterial protein extracts of the wild-type and Ssr54 mutant strains were prepared from logarithmic growth phase cultures, followed by two-dimensional PAGE. The same sample was tested in triplicate to confirm reproducibility of the two-dimensional electrophoresis maps. By comparing maps, we ascertained spots indicating highly differentially expressed proteins and conducted in-gel protease digestion, followed by mass spectroscopic analysis. Compared with the Ssr54 mutant strain, the wild-type strain had 137 differentially expressed protein spots. Protein spots with a difference in expression of at least three-fold were identified, subjected to robotic spot excision, trypsin digested, and subsequently analyzed using MALDI-TOF mass spectrometry. Protein identification was based on highly accurate masses and MS/MS sequence data from multiple tryptic peptides. Ssr54 deletion resulted in a total of 40 differentially expressed proteins, of which 25 were downregulated and 15 were upregulated (Supplementary Figure 2A). Supplementary Figure 2A shows the wild-type strain as per our previous results (Wang L. et al., 2016); however, when compared to the Ssr54 deletion strain, the protein spots differ. Twenty-six of the proteins differentially expressed in the wild-type and Ssr54 mutant strains were cytoplasmic, one was periplasmic, two were on the cytoplasmic leaflet of the plasma membrane, six were on the extracellular leaflet of the plasma membrane, and four were unidentified (Table 2).

After Ssr54 deletion, mRNA of proteins with the most significantly altered expression levels (TreF, OmpA [C43], and TolC) and their roles in stress tolerance and virulence were analyzed by qRT-PCR. The tolC and ompA genes were significantly downregulated in the Ssr54 mutant strain compared to those in the wild-type strain, whereas treF was significantly upregulated in the Ssr54 mutant strain.

To determine whether hyperosmotic conditions affect the expression of these target genes, treF, tolC, and ompA expression levels were determined under hyperosmotic conditions using qRT-PCR (Figure 8). Hyperosmotic conditions activated treF expression (1.99-fold) (P = 0.0115) and downregulated tolC and ompA expression (approximately two-fold) (P = 0.0207 and 0.0218, respectively).

### DISCUSSION

Numerous recent studies have investigated bacterial sRNAs, with more than 900 novel sRNAs reported in the sRNA database RNAMap (Huang et al., 2009). Of these, 79 were found in E. coli. However, there have been very few functional studies of sRNAs, and only 57 sRNA targets have been identified as...
Table 2 | Differentially expressed proteins in the sRNA mutant ΔSsr54 Shigella flexneri.

| Spot no. | NCBI GI identifier | Protein description | pI | Mr | Protein score | Final_Localization |
|----------|--------------------|---------------------|----|----|--------------|-------------------|
| **Downregulated protein spots in the ΔSsr54 mutant** | | | | | | |
| C04 | gi|56480278 | Polynucleotide phosphorylase | 5.35 | 55089 | 183 | Cytoplasmic |
| C05 | gi|24114698 | Glycogen branching protein | 5.91 | 55089 | 216 | Cytoplasmic |
| C06 | gi|56480212 | Transketolase | 5.48 | 55089 | 277 | Cytoplasmic |
| C08 | gi|24111559 | Pyruvate dehydrogenase dihydrolipoyltransacetylase | 5.09 | 55089 | 346 | Cytoplasmic |
| C09 | gi|24114835 | Glycyl-tRNA synthetase subunit beta | 5.29 | 55089 | 157 | Cytoplasmic |
| C13 | gi|24113658 | Bi-functional NAD/Hubiquinone oxidoreductase subunit C/D | 5.98 | 55089 | 132 | Cytoplasmic |
| C14 | gi|24112187 | ABC transporter ATP-binding protein | 4.99 | 55089 | 307 | Cytoplasmic |
| C20 | gi|56479850 | Dihydroxyacetone kinase subunit M | 4.66 | 55089 | 273 | Cytoplasmic |
| C21 | gi|24112268 | Seryl-tRNA synthetase | 5.34 | 55089 | 207 | Cytoplasmic |
| C22 | gi|56480244 | Outer membrane channel protein (Outer membrane channel TolC) | 5.35 | 55089 | 127 | Outer Membrane |
| C25 | gi|31983549 | Plasmid segregation protein (ParA) | 5.66 | 55089 | 384 | Cytoplasmic |
| C27 | gi|56480202 | Aromatic amino acid aminotransferase | 5.54 | 55089 | 113 | Cytoplasmic |
| C29 | gi|31983537 | Adenosine deaminase | 5.42 | 55089 | 208 | Cytoplasmic |
| C33 | gi|24114279 | Malate synthase G | 5.77 | 55089 | 198 | Cytoplasmic |
| C35 | gi|24112494 | Hypothetical protein SF1092 | 4.45 | 55089 | 204 | Cytoplasmic |
| C40 | gi|24114787 | Trehalase | 4.91 | 55089 | 196 | Cytoplasmic |
| C41 | gi|24112358 | Hydrogenase 1 large subunit | 5.51 | 55089 | 346 | Cytoplasmic |
| C42 | gi|24113658 | Pyruvate dehydratase | 5.82 | 55089 | 223 | Cytoplasmic |
| C43 | gi|24114255 | Outer membrane channel protein (Outer membrane channel TolC) | 5.58 | 55089 | 154 | Outer Membrane |
| C44 | gi|24112303 | Adenosine deaminase | 5.40 | 55089 | 208 | Cytoplasmic |
| C45 | gi|24112272 | Hypothetical protein SF0856 | 5.2 | 55089 | 188 | Unknown |
| C46 | gi|56479671 | Trehalose-6-phosphate phosphatase | 5.38 | 55089 | 258 | Unknown |
| C47 | gi|24112494 | Hypothetical protein SF1092 | 4.45 | 55089 | 453 | Cytoplasmic |
| C48 | gi|24113371 | DgsA anti-repressor MtfA | 4.63 | 55089 | 232 | Unknown |
| C49 | gi|24113539 | Methyl-galactoside ABC transporter substrate-binding protein MgIB(GBP) | 5.68 | 55089 | 242 | Periplasmic |
| C50 | gi|56479997 | Trehalose-6-phosphate phosphatase | 5.38 | 55089 | 258 | Unknown |
| C51 | gi|24112633 | Global DNA-binding transcriptional dual regulator H-NS | 5.43 | 55089 | 202 | Cytoplasmic |

| **Upregulated protein spots in ΔSsr54 mutant** | | | | | | |
| D03 | gi|24112479 | Malate synthase G | 5.77 | 55089 | 78 | Cytoplasmic |
| D05 | gi|24114787 | Trehalase | 4.91 | 55089 | 196 | Cytoplasmic |
| D06 | gi|24112358 | Hydrogenase 1 large subunit | 5.51 | 55089 | 346 | Cytoplasmic |
| D08 | gi|24112242 | Pyruvate dehydratase | 5.82 | 55089 | 223 | Cytoplasmic |
| D12 | gi|24114255 | Outer membrane channel protein (Outer membrane channel TolC) | 5.58 | 55089 | 154 | Outer Membrane |
| D33 | gi|24113013 | Adenosine deaminase | 5.40 | 55089 | 208 | Cytoplasmic |
| D35 | gi|56479781 | Outer membrane protein OmpA | 5.65 | 55089 | 63 | Outer Membrane |
| D36 | gi|24111549 | Guanosine 5'-monophosphate oxidoreductase | 5.1 | 55089 | 124 | Cytoplasmic |
| D40 | gi|24112494 | Hypothetical protein SF1092 | 4.45 | 55089 | 453 | Cytoplasmic |
| D41 | gi|24113371 | DgsA anti-repressor MtfA | 4.63 | 55089 | 232 | Unknown |
| D44 | gi|24113539 | Methyl-galactoside ABC transporter substrate-binding protein MgIB(GBP) | 5.68 | 55089 | 242 | Periplasmic |
| D54 | gi|56479781 | Outer membrane protein OmpA | 5.65 | 55089 | 152 | Outer Membrane |

*pl represents the isoelectric point of the protein sample; Mr represents the molecular mass of the protein sample.*

In the present study, we confirmed the presence of a novel sRNA, Ssr54, in *S. flexneri*, observed its role in environmental stress tolerance and virulence, and identified possible targets to suggest mechanisms of action.

In pathogenic bacteria, response to osmotic stress is a critical mechanism for survival during environmental osmotic stress as well as for establishing infection. For instance, the ProU system functions to allow *S. sonnei* to tolerate hyperosmotic stress in...
vitro, while also facilitating the survival and proliferation of the bacteria within stressful intracellular niches (Mahmoud et al., 2017). Herein, we observed that when exposed to hyperosmotic stress, Ssr54 was downregulated and survival of the Ssr54 deletion strain increased. Generally, bacteria tolerate changes in osmolality by solute efflux or gene regulation (Mahmoud et al., 2017), allowing them to adapt for survival. In this way, the downregulation of Ssr54 may serve as a protective mechanism for S. flexneri in the gastrointestinal tract. Furthermore, Ssr54 deletion was shown to upregulate expression of the target gene treF. TreF, the cytosolic trehalase, is weakly induced by high osmolality of the medium and is partially dependent on RpoS, the stationary-phase sigma factor. Hence, treF is expressed primarily under hyperosmotic conditions (Horlacher et al., 1996; Weber et al., 2006); accordingly, Ssr54 expression seems to inhibit treF, with treF upregulated under high osmotic concentrations when Ssr54 is repressed. Ssr54 may, therefore, upregulate treF to enhance survival under osmotic stress. Thus, virulence genes in Shigella are expressed in response to increases in osmolality.

Shigella guinea pig Sereny tests, lung invasion assays, and HeLa invasion assays were also performed to determine whether Ssr54 regulates S. flexneri virulence. Results suggest that the virulence of S. flexneri ΔSsr54 was significantly decreased as compared to that of the wild-type strain. TolC is an outer membrane channel protein that contributes to bacterial adhesion, antibiotic resistance, and invasion during disease pathogenesis (Stone and Miller, 1995; Shaffer et al., 2001; Platz et al., 2010). Ssr54 deletion downregulated tolC, which may have attenuated the virulence of S. flexneri. Our study also showed that deletion of Ssr54 downregulated ompA. OmpA, the major outer membrane protein, plays important roles in the virulence of pathogens (Scribano et al., 2016). Some studies have suggested that micA and rseX sRNAs bind to the ompA translation initiation region and block ribosome binding to prevent expression of ompA in E. coli (Udekwu et al., 2005). Hence, we speculate that ompA was downregulated in the Ssr54 deletion strain, decreasing the virulence of S. flexneri. Overall, Ssr54 may affect S. flexneri virulence by regulating the expression of putative target genes tolC and ompA, either directly or indirectly.

Studies have suggested that hyperosmotic pressures affect virulence gene expression in S. flexneri (Porter and Dorman, 1994). Under hyperosmotic stress, the hyperosmotic regulator gene treF was upregulated, whereas the virulence regulator genes tolC and ompA were downregulated, indicating that Ssr54 regulates treF to increase S. flexneri tolerance to hyperosmotic conditions and regulates virulence genes tolC and ompA to reduce pathogenicity of this bacterium. These results have, therefore, established the role of Ssr54 in survival of Shigella under hyperosmotic environments; however, whether it is required in the context of infection remains unknown. Moreover, whether Ssr54 regulates virulence and hyperosmotic stress tolerance by regulating genes directly, or via other pathways, remains to be answered; we are currently investigating this mechanism.

DATA AVAILABILITY STATEMENT

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for Animal Experimentation of the Academy of Military Medical Sciences.

AUTHOR CONTRIBUTIONS

HS, LW, and HM designed the research, assessed and interpreted the results, and prepared the manuscript. GY, BL, LJ, HQ, MY, and BZ carried out the data analysis and designed the experiments. JX, SQ, and PL assisted in carrying out the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00483/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.