Identification and Characterization of a Cis Antisense RNA of the \textit{rpoH} Gene of \textit{Salmonella enterica} Serovar Typhi

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Antisense RNAs from complementary strands of protein coding genes regulate the expression of genes involved in many cellular processes. Using deep sequencing analysis of the \textit{Salmonella enterica} serovar Typhi (S. Typhi) transcriptome, a novel antisense RNA encoded on the strand complementary to the \textit{rpoH} gene was revealed. In this study, the molecular features of this antisense RNA were assessed using northern blotting and rapid amplification of cDNA ends. The 3,508 nt sequence of RNA was identified as the antisense RNA of the \textit{rpoH} gene and was named ArpH. ArpH was found to attenuate the invasion of HeLa cells by S. Typhi by regulating the expression of SPI-1 genes. In an \textit{rpoH} mutant strain, the invasive capacity of S. Typhi was increased, whereas overexpression of ArpH positively regulates \textit{rpoH} mRNA levels. Results of this study suggest that the \textit{cis}-encoded antisense RNA ArpH is likely to affect the invasive capacity of S. Typhi by regulating the expression of \textit{rpoH}.

Keywords: \textit{Salmonella enterica} serovar Typhi, antisense RNA, ArpH, \textit{rpoH}, invasion

INTRODUCTION

\textit{Salmonella enterica} serovar Typhi (S. Typhi) is an important Gram-negative pathogenic bacterium that can cause symptoms ranging from mild diarrhea to serious systemic infections, such as typhoid fever, owing to many virulence genes (Dougan and Baker, 2014). Concentrated areas of virulence genes, called \textit{Salmonella} pathogenicity islands (SPI), are located on the bacterial chromosome, plasmids, or phages (Matsui et al., 2008). The majority of virulence genes are located on SPIs on the chromosome. Virulence genes protect bacteria from damage caused by the host immune system. However, S. Typhi also interacts with the host and changes the host cell environment. The ability of S. Typhi to invade and survive in non-phagocytic cells is an indication of its pathogenicity. These pathogenic mechanisms have been linked to SPIs (Nieto et al., 2016).

Pathogens can quickly adapt to changing circumstances by regulating expression of their virulence genes during the process of infection. Many non-coding RNAs (ncRNAs) involved in complex regulatory mechanisms have been identified. Research has demonstrated that ncRNAs are involved in transcriptional and post-transcriptional regulation via the formation of complexes with DNA or proteins through base pairing interactions (Kaikkonen et al., 2011; Ghosal et al., 2013). Previously, ncRNA was considered to be transcriptional noise that was not involved in protein coding; however, a growing number of studies have confirmed that ncRNA can play a
role in regulating the expression of many genes in bacteria, including ABC transport system (Miyakoshi et al., 2015), quorum sensing (Bardill and Hammer, 2012), oxidative stress (Gerstle et al., 2012), acid resistance (Aiso et al., 2011), and virulence genes. ncRNAs are classified as either trans-encoded or cis-encoded based on their position in the genome. Trans-encoded ncRNAs are located between protein coding genes and, because they are located at distant genomic locations, are only partially complementary to their target mRNAs and act via incomplete base pairing. Cis-encoded ncRNAs are located within protein-coding genes and, because they are located in the same region, are fully complementary to their target mRNAs (Storz et al., 2011).

We previously conducted deep RNA sequencing analysis of the transcriptome and identified several novel cis-encoded antisense RNAs (Dadzie et al., 2013, 2014). A 1164-nt transcript partially encoded by the minus strand of rpoH was expressed. The maximum expression of the transcript is from 1887 nt downstream from the start codon of yhhK and overlaps the 192-nt region upstream from the rpoH gene start codon, as shown by bioinformatic prediction (Figure 1A). RpoH activates expression of the hfg2 gene, which encodes an RNA-binding regulatory protein. RpoH also regulates the expression of genes involved in adaptations to environmental stresses, such as thermal stress (Martinez-Salazar et al., 2009; López-Leal et al., 2016). However, the full length and function of the antisense RNA of rpoH is not yet known. In the present study, we described the identification and characterization of a novel antisense RNA encoded by the strand complementary to the rpoH gene sequence that we named ArpH. We demonstrated that ArpH is likely to affect the invasive capacity of S. Typhi by regulating the expression of rpoH.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The strains and plasmids used in this study are listed in Table 1. All strains were grown at 37°C in Luria-Bertani (LB) medium supplemented with 100 µg/mL ampicillin or 50 µg/mL kanamycin when required.

Strain Construction

The oligonucleotides used in this study are listed in Table 2. To construct the arpH mutant strain, primer pairs F1A/F1B and F2A/F2B were used to amplify fragments F1 (467 bp) and F2 (409 bp), which are located 355 nt upstream and 80 nt downstream of the arpH gene transcriptional start site, respectively. F1 and F2 were used as templates for a second PCR using primers F1A/F2B to obtain a single 876 bp fragment. BamHI sites were added to the 5'-ends of primers F1A and F2B. The 876 bp fragment was then inserted into the BamHI site of the suicide plasmid pGMB151, carrying the sucrose-sensitivity gene sacB. pGMB151 containing the insert was electroporated into the S. Typhi wild-type strain. Putative arpH mutants were cultured on LB plates containing sucrose. Colonies were screened for inserts by PCR with primers F1A/F2B and by DNA sequencing and mutants were named ΔarpH (Huang et al., 2004).

An rpoH mutant was constructed using the lambda Red recombinase method (Datsenko and Wanner, 2000). A kanamycin resistance (kan) gene was amplified from the pET-28a plasmid using primers rpoHF1A/F1B. The kan sequences in the amplified fragment were flanked with 50 bp sequences complementary to either end of the rpoH promoter region. The deletion fragment is located 171 nt upstream and 60 nt downstream of the rpoH gene transcriptional start site. Electrocompetent cells containing the plasmid pKD46 were transformed with the purified PCR product. The transformants were cultured on LB plates containing kanamycin. rpoH mutant strains were screened by PCR using the primers rpoHF2A/F2B and rpoHF2A/F1B and the mutants named ΔrpoH.

Plasmid Construction

To construct the plasmid pBAD-arpH, primers PA and PB were used to amplify a 1,182 bp fragment of arpH, which consists of 1164bp transcript and 18bp of restriction fragment.
(Figure 1A). The PCR product was cloned into an NcoI/HindIII-digested pBAD/Myc-His A vector (Invitrogen). The wild-type S. Typhi and ΔarpH strains were transformed with the recombinant vector pBAD-arpH or the pBAD plasmid alone using electroporation to generate the strains WT-pBAD-arpH, WT-pBAD, ΔarpH-pBAD-arpH, and ΔarpH-pBAD. RNase E and RNase III mutants were electroporated with pBAD-arpH to obtain Δrne-arpH and Δrnc-arpH (Dadzie et al., 2013).

**RNA Extraction**

Salmonella enterica serovar Typhi grown overnight in LB broth was diluted 1:100 and grown at 37°C with shaking at 250 rpm. Total RNA was extracted at OD_{600} = 0.8 using the RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol for assessment of the presence of ArpH. For overexpression analysis, S. Typhi and ΔarpH carrying pBAD or pBAD-arpH plasmids were grown in LB broth until the OD_{600} reached 0.4 and induced by the addition of 0.2% l-arabinose. The cells were harvested by centrifugation at 10,000 × g at 4°C. Total RNA was then extracted using TRIzol Reagent (Invitrogen) followed by treatment with RNase-free DNase I (Takara). RNA was quantified using spectrophotometer at 260 nm.

**5′- and 3′-RACE Analysis**

We performed 5′ rapid amplification of cDNA ends (5′-RACE) experiments as previously described (Zhang et al., 2015). A total of 10 units of calf intestine alkaline phosphatase (TakaRa) was added to 5 μg total RNA for 60 min at 50°C. One unit of tobacco acid pyrophosphatase (TakaRa) was then added for 60 min at 37°C. Total RNA was ligated using T4 RNA ligase (TakaRa) for 60 min at 16°C. cDNA was synthesized using M-MLV reverse transcriptase (TakaRa) according to the manufacturer’s instructions with an antisense-specific primer (5′RACE RT). The first round of PCR amplification was performed using a 5′-RACE adaptor-specific primer (5′RACE outer primer) and an arpH-specific primer (5′RACE GSP1). A second round of PCR amplification was performed using the primers 5′RACE inner primer and 5′RACE GSP2. The PCR products were extracted, subcloned, and sequenced to confirm the transcription initiation site of the arpH sequence.
3′-RACE was carried out using a SMARTer RACE kit (Takara) according to the manufacturer’s instructions. Total RNA was treated with calf intestine alkaline phosphatase (Takara) and ligated to the 5′ RNA was treated with calf intestine alkaline phosphatase (Takara) according to the manufacturer’s instructions. Total hybridized overnight at 42°C (Roche). Membranes were prehybridized in Rapid-Hyb buffer probes were synthesized using a DIG Northern Starter kit 3′ primers (3′RACE adaptor primer) complementary to the 3′RACE adaptor. The outer and inner PCR reactions were performed using the 3′-RACE adaptor primer and arpH-specific primers (3′RACE GSP1 and 3′RACE GSP2). PCR amplification, cloning, and sequencing were performed as described for 5′-RACE.

**Northern Blotting Analysis**

To detect ArpH, an arpH-specific cDNA probe was used, which was located from 130 nt to 183 nt downstream of the rpoH gene initiation codon on the complementary strand (Figure 1). A total of 10–20 μg of total RNA was separated on a 6% polyacrylamide gel containing 7 M urea and electroblotted onto HybondTM N+ membranes (GE Healthcare). The cDNA probes were synthesized using a DIG Northern Starter kit (Roche). Membranes were prehybridized in Rapid-Hyb buffer (GE Healthcare). The digoxigenin-labeled riboprobes were hybridized overnight at 42°C with the prehybridized membranes. Membranes were washed as previously described (Zhang et al., 2015). Riboprobed membranes were exposed to KODAK x-ray film at −70°C.

**Quantitative Real-Time PCR**

Equal amounts of RNA were reverse transcribed into cDNA using random primers (Takara). The primer pairs used for quantitative real-time PCR (qRT-PCR) analysis are shown in Table 2. Reactions were monitored using a C1000 Thermal Cycler (Bio-Rad) and transcript levels were normalized to 5S ribosomal RNA using the 2−ΔΔCt method (Bader et al., 2003). Each experiment was performed according to the manufacturers’ protocols in triplicate.

**Growth Curves**

Strain growth was determined by measuring the OD600 using a BioPhotometer (Eppendorf). WT-pBAD and WT-pBAD-arpH strains were grown overnight in LB broth containing 100 mg/mL ampicillin, diluted 1:100, and incubated at 37°C with shaking at 250 rpm with the addition of 0.2% l-arabinose. To monitor the growth rate, cells were cultured in LB to an OD600 of 0.4 under acid stress (HCl, pH 4.5), oxidative stress (1 mM H2O2), or osmotic stress (0.3 M NaCl) conditions. The absorbance was measured at 1 h intervals for 24 h and growth curves were determined from biological triplicates.

**Microarray Analysis**

For gene expression profiling experiments, single colonies of WT-pBAD and WT-pBAD-arpH grown overnight in LB broth containing 100 mg/mL ampicillin were diluted 1:100 and incubated at 37°C with shaking at 250 rpm with 0.2% l-arabinose. Cells were then cultured in LB to an OD600 of 0.4 and treated with 1 mM of H2O2 for 4 h. Total RNA was extracted using the RNeasy Mini kit (Invitrogen) according to the manufacturer’s protocol. Samples were harvested by centrifugation at 10,000 × g at 4°C. Total RNA was isolated using TRIzol Reagent (Invitrogen) followed by treatment with RNase-free DNase I (Takara). A total of 25 μg RNA was used as the template for cDNA synthesis. Genomic microarray analysis of S. Typhi was performed as described previously (Sethi et al., 2010; Du et al., 2011). The microarray was analyzed by comparing spot intensities of WT-pBAD and WT-pBAD-arpH strains (Zhang et al., 2009).

**Epithelial Cell Invasion Assay**

HeLa cells (2 × 10⁵) were seeded into 24-well tissue culture plates containing RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum and incubated at 37°C in 5% CO2 for 16 h. S. Typhi cultures were grown to an OD600 of 0.4. The bacterial cells were washed thoroughly with phosphate-buffered saline at a multiplicity of infection of 20. The monolayers were either lysed with Triton X-100 to evaluate the level of adherence (T0) or incubated for a further 3 h in medium containing 100 μg/mL gentamicin to eliminate extracellular bacteria and to assess the level of invasion (T90). The invasiveness of S. Typhi was previously demonstrated by our laboratory (Gong et al., 2015).

**Statistical Analysis**

Results are presented as mean ± SD. Significant differences between groups were assessed using Student’s t-tests or analysis of variance using SPSS software (SPSS Inc.). Differences were considered significant at P < 0.05.

**RESULTS**

**Identification and Expression of ArpH**

Recently, several new ncRNAs were identified by deep sequencing analysis of the S. Typhi genome (Dadzie et al., 2013, 2014; Gong et al., 2015). The RNA-seq analysis of S. Typhi under stress conditions showed a 1164-nt transcript partially encoded by the minus strand of rpoH, extending from 192 nt upstream to 972 nt downstream from the start codon of rpoH (Figure 1A). In the current study, total RNA was extracted from a wild-type S. Typhi strain and hybridized with an arpH-specific digoxigenin-labeled cDNA probe to confirm the existence of ArpH. We found that the arpH fragment was approximately 3,000 nt (Figure 1B). 5′-RACE was then performed to map the 5′-end of the arpH sequence, which was found to be 411 nt upstream of the yhhK initiation codon (Figure 1A). 3′-RACE revealed that the 3′-end of the arpH sequence was 238 nt upstream of the rpoH initiation codon on the complementary strand. The results of RACE analyses indicated that the total length of the arpH sequence is 3508 nt.

We monitored the levels of ArpH over time and under different stress conditions using northern blotting analysis and qRT-PCR. Total RNA was harvested from wild-type S. Typhi grown to an OD600 of 0.3, 0.8, 1.3, or 2.0, representing the lag phase through the stationary phase (Figure 2A). The expression
of ArpH was also measured by qRT-PCR (Figure 2B). To examine levels of ArpH under stress conditions, we simulated the environment that Salmonella encounters upon invasion or within macrophages. Total RNA was extracted after cells were subjected to acid, osmotic, or oxidative stress. Northern blotting and qRT-PCR demonstrated that ArpH levels were lower when S. Typhi was exposed to acidic conditions and higher when exposed to oxidative conditions compared to S. Typhi grown under normal conditions (Figures 3A,B). These results indicate that ArpH is expressed in S. Typhi and that its expression changes in response to stress conditions.

**Effect of ArpH Overexpression on rpoH mRNA Levels**

To examine the effect of ArpH on its putative mRNA target rpoH, we investigated expression levels of rpoH mRNA using qRT-PCR after overexpressing the partial arpH sequence from the arabinose-inducible recombinant plasmid WT-pBAD-arpH. The partial overexpression of the arpH did not affect the expression of the yhhK, livJ, and rpoH gene, which were estimated with qRT-PCR (data not shown). The relative level of ArpH in WT-pBADarpH strain was significantly higher than the WT-pBAD strain by qRT-PCR (data not shown), which were consistent with our expectation. In contrast to WT-pBAD alone, the mRNA level of rpoH increased gradually within 20 min of ArpH overexpression (Figures 4A,B). These results indicate that ArpH positively regulates rpoH mRNA levels.

RNase E and RNase III are the main endoribonucleases that cleave antisense RNA-induced target mRNA (Saramago et al., 2014). RNase E cuts RNA at a number of single-stranded regions, whereas RNase III cleaves double-stranded RNA (Arraiano et al., 2010). To determine the effect of ArpH overexpression on rpoH mRNA levels in RNase E and RNase III mutants, we constructed Δrne-arpH and Δrnc-arpH strains. The mRNA expression of rpoH was sixfold higher in RNase III mutants than in the control strains (Figure 4C), whereas the levels were relatively similar in RNase E mutants, indicating that RNase III may play a more significant role in the coupled degradation of the ArpH/RpoH duplex.

**Effect of ArpH Overexpression on S. Typhi Growth**

We investigated the growth of WT-pBAD and WT-pBADarpH strains over a 24 h period. Figure 5A shows that the growth curves of the two strains were similar during the lag and stationary phases; however, during the logarithmic phase, the WT-pBAD-arpH strain grew slightly faster than the WT-pBAD strain. Growth curves were also constructed for the WT-pBAD and WT-pBAD-arpH strains under different growth conditions. The growth curves for WT-pBAD and WT-pBAD-arpH strains were similar under osmotic stress. However, the growth of the WT-pBAD-arpH strain was enhanced under oxidative stress during the early logarithmic phase and overexpression of ArpH resulted in significant reduction of bacterial growth under acid stress during the late logarithmic phase (Figures 5B–D). Additionally, the growth curves of the WT-pBAD and WT-pBAD-arpH strains were similar under thermal stress (data not shown).
using a whole genome microarray to investigate the influence of ArpH overexpression in S. Typhi under oxidative stress conditions (Supplementary Material). Microarray results showed differential expression of genes involved in SPI-1 and invasion, flagellar biosynthesis, and virulence between the WT-pBAD-arpH and WT-pBAD strains. SPI-1 and invasion-associated genes, including **prgH, iagA, sipCDA, invFGA, spaKINM,** and **tviACDE,** were downregulated in the WT-pBAD-arpH strain. Metabolism-associated genes, including **astB, msyB, glpD, argD,** and **sdhDC,** were also downregulated in the WT-pBAD-arpH strain. Expression of flagellum-associated genes, such as **flgBCDEF, fliACJHLSZ,** and **flhDC,** were not significantly different in the WT-pBAD-arpH strain. However, the superoxide dismutase gene **sodB** and the oxygen-regulated invasion gene **orgA** were upregulated in the WT-pBAD-arpH strain. Using qRT-PCR, we confirmed the microarray results by comparing the expression of the genes **sodB, orgA, prgH,** and **sipA** between the WT-pBAD-arpH and WT-pBAD strains (Figure 6). These findings suggested that ArpH plays a role in the antioxidant defenses of S. Typhi under oxidative stress conditions (Bang et al., 2005).

**arpH Deletion Enhances the Invasion of Epithelial Cells by S. Typhi**

We constructed an arpH mutant in which the 435-nt arpH fragment was disrupted, whereas the SD box and the ORF structure of yhhK, livJ, and rpoH were retained intact. The deletion of the arpH did not affect the expression of the yhhK, livJ, and rpoH gene, which were estimated with qRT-PCR (data not shown), and the results were consistent with our expectation. An rpoH deletion mutant was also constructed using the lambda Red recombinase method. Because the arpH-encoding region overlaps the rpoH gene and the arpH might influence RpoH function, we investigated the function of rpoH in this study. A rpoH mutant was constructed, in which the deleted region was only the promoter region of rpoH. The deletion of the rpoH did not affect the expression of the arpH gene, which was estimated with qRT-PCR (data not shown), and found to be consistent with our expectation. To examine the possibility that ArpH may play a role in the invasion of epithelial cells by Salmonella, we examined the invasion efficiency of wild-type, DeltaarpH, and DeltaarpH strains into HeLa cells. To confirm these findings, we used qRT-PCR to measure the mRNA levels of the SPI-1 and invasion-associated genes **prgH, sipA,** and **invF** (Figure 7A). From the results shown in Figure 7B, we concluded that the invasion efficiency of the DeltaarpH strain was significantly higher than that of the wild-type strain. Taken together, these findings indicate that ArpH on RpoH might negatively regulate the invasion of S. Typhi into the intestinal epithelium.

**rpoH Deletion Upregulates Expression of Invasion-Associated Genes**

Transcriptome analysis of the rpoH mutant was carried out using a whole genome microarray. Significant upregulation of the invasion-associated genes **prgH, sipA,** and **invF** was observed in the DeltaarpH strain. In contrast, no significant increase in the
expression levels of these genes was observed in the wild-type strain. To confirm the findings of the microarray experiment, we used qRT-PCR to measure the expression of the prgH, sipA, and invF genes (Figure 7A). The HeLa cell invasion assay demonstrated that the invasion capacity of the ΔrpoH strain was dramatically enhanced compared to the wild-type strain. This result is consistent with previous literature (Matsui et al., 2008). However, the results of the invasion assay demonstrated that there was no significant difference in invasion capacity between the ΔarpH and ΔrpoH mutants (Figure 7B). Taken together, these findings suggest that ArpH might downregulate the invasiveness of S. Typhi by affecting the expression of rpoH.

**Effect of ArpH Overexpression on Invasiveness of S. Typhi**

In order to further study the mechanism by which ArpH affects the invasiveness of S. Typhi, the wild-type S. Typhi and ΔrpoH strains were transformed with the recombinant vector pBAD-arpH or the pBAD plasmid alone using electroporation to generate the strains WT-pBAD-arpH, WT-pBAD, ΔrpoH-pBAD-arpH, and ΔrpoH-pBAD. We then used qRT-PCR to measure the mRNA levels of the invasion-associated gene invF and examined the invasion efficiency of these strains into HeLa cells. We concluded that the mRNA levels of invF and invasion efficiency of the WT-pBAD-arpH strain were lower than that of the WT-pBAD strain. However, when ArpH was overexpressed in the rpoH mutant, mRNA levels of invF and invasion into HeLa cells were not significantly different from the rpoH mutant alone (Figure 8). These results seem to indicate that the effect of ArpH on the invasiveness of S. Typhi may be due solely to its effects on rpoH. Additionally, the difference of twofold in the HeLa cell invasion assay may be statistically calculated to be significant, but in reality from a biological perspective it is probably almost insignificant. Therefore, the effect of ArpH on the invasiveness of S. Typhi needs further research to exclude experimental variation.

**DISCUSSION**

Many ncRNAs are involved in regulating the expression of genes involved in different biological processes through a variety of mechanisms (Loh et al., 2012; Chew et al., 2018). We applied deep sequencing analysis to discover ncRNAs in bacteria, including those involved in the invasion of epithelial cells by Salmonella (Dadzie et al., 2013, 2014; Gong et al., 2015; Zhang et al., 2015). Recently, novel ncRNAs associated with the response to stress were identified (Gong et al., 2011, 2015). By combining the
findings of northern blotting and RACE analyses, we identified the cis-encoded ncRNA ArpH, which is complementary to rpoH mRNA. The full-length antisense RNA transcript was 3,508 nt from 411 nt upstream of the yhhK gene initiation codon to 238 nt upstream of the rpoH gene initiation codon on the complementary strand (Figure 1). No open reading frame region longer than 150 nt or Shine–Dalgarno sequence except for yhhK was identified in the entire sequence. Therefore, we believe that ArpH is a new ncRNA. It is worth mentioning that the yhhK gene might play an ancillary role in pantothenate biosynthesis in S. enterica (Stuecker et al., 2012). Because the arpH-encoding region overlaps the yhhK gene and the arpH might influence YhhK function. We constructed ArpH deletion mutant and overexpression strains away from yhhK coding region, which were estimated with qRT-PCR (Supplementary Figures S1, S2). In addition, the livJ gene is a pseudogene in S. Typhi.

We performed northern blotting and qRT-PCR to determine the expression of ArpH in S. Typhi. Peak expression of ArpH occurred in the late logarithmic phase (Figure 2). ArpH expression was reduced in acid stress conditions and increased in oxidative stress conditions (Figure 3). Based on these results, we investigated the effect of ArpH expression on bacterial growth. Growth curves of wild-type and ArpH overexpressing strains were similar during the lag and stationary phases. However, during the logarithmic phase, when the levels of endogenous ArpH transcripts were highest, the overexpression strain grew slightly more quickly than the wild-type strain (Figure 5A). Growth curves were also generated for WT-pBAD and WT-pBAD-arpH strains under different growth conditions. The growth curves of both strains were similar under osmotic stress. However, the growth of the WT-pBAD-arpH strain was enhanced under oxidative stress during the early logarithmic phase and overexpression of ArpH resulted in significant reduction of bacterial growth under acid stress during the late logarithmic phase (Figures 5B–D), indicating that the expression of ArpH enhances growth under oxidative stress conditions (Bang et al., 2005; Nuss et al., 2009; Remes et al., 2017). These findings are in accordance with the observation that, in general, ncRNAs are activated in response to different environmental conditions and that they function to help cells adapt to stress (Waters and Storz, 2009).

ncRNAs can regulate the translation of target genes by altering the stability of mRNAs (Ayupe and Reis, 2017). To clarify the effect of ArpH on its putative mRNA target rpoH, we investigated the expression levels of rpoH mRNA using qRT-PCR after overexpressing the partial arpH sequence from the arabinose-inducible recombinant plasmid WT-pBAD-arpH. rpoH mRNA levels increased gradually within 20 min of ArpH overexpression.
Microarray experiments showed that genes involved in SPI-1 and invasion, flagellar biosynthesis, and virulence were differentially expressed between WT-pBAD-arpH and WT-pBAD strains under oxidative stress conditions (see Supplementary Material). The superoxide dismutase gene sodB and the oxygen-regulated invasion gene orgA were upregulated in the WT-pBAD-arpH strain (Figure 6). The orgA gene is involved in promoting cellular invasion of the pathogen. Previously published work has indicated that the prgH, -I, -J, and -K genes are transcribed from a promoter distinct from that used by the gene immediately downstream, orgA (Klein et al., 2000). However, its exact role in virulence is still unclear mainly due to difficulties in understanding its complex regulation. Previous studies have been consistent regarding oxygen regulation of orgA (Russell et al., 2004). The reason may be that orgA is mainly regulated by oxidative stress. This suggests that ArpH may be involved in regulating the anti-oxygenation of S. Typhi under oxidative stress conditions (Gläser et al., 2011). These findings are in agreement with the fact that the WT-pBAD-arpH strain grew more quickly than the WT-pBAD strain under oxidative stress conditions. Taken together, these data suggested that ArpH played a role in the antioxidant defenses of S. Typhi under oxidative stress conditions. These results are consistent with literature (Bang et al., 2005; Matsui et al., 2008). The microarray data revealed that the expression of flagellum-associated genes, such as flgBCDEF, fliA, fliHLSZ, and flhDC, were not significantly different in the WT-pBAD-arpH strain (see Supplementary Material). This indicates that ArpH does not participate in the regulation of S. Typhi motility. In addition, no significant changes were observed in the motility phenotypes of the WT-pBAD-arpH or WT-pBAD strains in motility agar (data not shown), which is in agreement with the microarray data.

A type III secretion system encoded by SPI-1 mediates the invasion of epithelial cells by Salmonella (Hensel, 2004). The deletion of ArpH increased bacterial invasion compared to wild-type cells (Figure 7). And the mRNA levels of invF and invasion efficiency of the WT-pBAD-arpH strain were lower than that of the WT-pBAD strain (Figure 8). This corresponds to the microarray results, which showed that invasion-associated genes, including prgH and sipA, were downregulated when ArpH was overexpressed (Figure 6). Our research is the first to demonstrate that ArpH is a player in host cell invasion by S. Typhi. It was previously reported that RpoH regulates virulence in many bacteria, including Salmonella (Bang et al., 2005; Delory et al., 2006; Grall et al., 2009; De la Cruz et al., 2016). Additionally, RpoH negatively regulates SPI-1 expression (Matsui et al., 2008). Due to the increase in expression of invasion-related genes, and in bacterial invasion observed in both arpH and rpoH deletion mutants, we hypothesize that ArpH may affect Salmonella invasiveness by acting on rpoH (Figure 7). When ArpH was overexpressed in rpoH mutants, the mRNA levels of invF and the invasion efficiency into HeLa epithelial cells were not significantly different from wild-type cells (Figure 8). These results seem to indicate that the effect of ArpH on the invasiveness of S. Typhi may be due solely to rpoH. We hypothesize that the mechanism by which ArpH affects Salmonella invasiveness is through the activation of rpoH expression (Bang et al., 2005; Matsui et al., 2008). However, while rpoH may have a small effect compared with WT-pBAD (Figures 4A,B). These results indicate that overexpression of ArpH positively regulates rpoH mRNA levels. To further determine the effect of ArpH overexpression on rpoH mRNA levels, we constructed RNase E and RNase III mutants. Expression levels of rpoH mRNA increased significantly in RNase III mutants (Figure 4C), whereas levels were relatively similar in RNase E mutants, indicating that RNase III may play a more significant role in the coupled degradation of the ArpH/RpoH duplex (Nicholson, 2014). Although some antisense RNAs inhibit target sense mRNA expression, many sense-antisense RNA pairs promote coordinated expression, suggesting that antisense RNAs may be involved in regulating the stability of cis-encoded mRNAs (Chinni et al., 2010; Lee and Groisman, 2010).
on the invasion phenotype, there are several confusing details about how arpH alters transcription of invasion genes, therefore, further detailed research is needed.

CONCLUSION

The full-length arpH sequence was found to be 3,508 nt located 411 nt upstream of the yhhK initiation codon and 238 nt upstream of the rpoH initiation codon on the complementary strand. ArpH is likely to affect the expression of rpoH in cis at the transcriptional level.

AUTHOR CONTRIBUTIONS

CX and XH conceived and designed the experiments. CX, XL, XZ, and JL performed the experiments. CX, XL, and XH analyzed the data. CX, SX, and XL contributed reagents, materials, and analysis tools. CX and XH wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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