HmsB enhances biofilm formation in *Yersinia pestis*

Nan Fang†, Shi Qu†, Huiying Yang, Haihong Fang, Lei Liu, Yiquan Zhang, Li Wang, Yanping Han, Dongsheng Zhou* and Ruifu Yang*

State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China

**INTRODUCTION**

*Yersinia pestis* is the causative agent of plague, one of the most dangerous infectious diseases. Flea-borne transmission of *Y. pestis* occurs among mammals including humans, which distinguishes this pathogen from its genetically close progenitor *Y. pseudotuberculosis* that is a mild food-borne enteric pathogen (Zhou and Yang, 2011). *Y. pestis* biofilms, a population of bacterial colonies embedded in a self-produced exopolysaccharide matrix (Darby, 2008; Hinnebusch and Erickson, 2008; Zhou and Yang, 2011), can attach to and physically block flea’s proventriculus. The inability to take in a blood meal when the proventriculus is blocked makes fleas feel hungry and bite repeatedly and thereby promoting *Y. pestis* to be spread into new individuals of mammalian reservoirs (Darby, 2008; Hinnebusch and Erickson, 2008; Zhou and Yang, 2011).

*Yersinia pestis* biofilms can also block feeding of model nematode *Caenorhabditis elegans*, because attached biofilms are primarily found on the larva head to blanket the mouth (Darby et al., 2002; Fang et al., 2013). By contrast, most strains of *Y. pseudotuberculosis* have the biofilm-negative phenotype, although a few of them (being similar to *Y. pestis*) can form robust biofilms at gas-liquid-solid interfaces or on nematodes (Erickson et al., 2006; Fang et al., 2013).

The hmsHFRS operon is responsible for biosynthesis and translocation of biofilm matrix exopolysaccharide. *Yersinia pestis* expresses the two sole diguanylate cyclases HmsT and HmsD and the sole phosphodiesterase HmsP which are specific for biosynthesis and degradation, respectively, of 3′,5′-cyclic diguanosine monophosphate (c-di-GMP), a second messenger promoting exopolysaccharide production. In this work, the phenotypic assays indicates that *Y. pestis* sRNA HmsB enhances the production of c-di-GMP, exopolysaccharide, and biofilm. Further gene regulation experiments disclose that HmsB stimulates the expression of hmsB, hmsCDE, hmsT, and hmsHFRS but represses that of hmsP. HmsB most likely acts as a major activator of biofilm formation in *Y. pestis*. This is the first report of regulation of *Yersinia* biofilm formation by a sRNA. Data presented here will promote us to gain a deeper understanding of the complex regulatory circuits controlling *Yersinia* biofilm formation.

**Keywords:** *Yersinia pestis*, HmsB, c-di-GMP, biofilm
mutant of *Y. pestis* (Table 1). For *in trans* complementation, a PCR-generated DNA fragment containing the coding region of each indicated gene together with its promoter-proximal region and transcriptional terminator-proximal region was cloned into the cloning vector pBluescript II KS(+) (Agilent Technologies). The resulting recombinant vector was transformed into indicated *Y. pestis* strain lack of the corresponding functional gene, generating the corresponding complemented mutant (Table 1). All the primers designed in this study were listed in Table S1.

### BACTERIAL GROWTH AND RNA ISOLATION

Overnight cell cultures in the Luria-Bertani (LB) broth with an optical density (OD\textsubscript{620}) of about 1.0 were diluted 1:50 into 18 ml of fresh LB broth for further cultivation at 26°C with shaking at 230 rpm to reach the middle stationary phases (an OD\textsubscript{620} of 0.8–1.2), followed by cell harvest for further gene regulation or phenotypic assays. Immediately before bacterial harvest for RNA isolation, double-volume of RNAprotect reagent (Qiagen) was added to one-volume of cell culture, and total RNA was extracted using TRIzol Reagent (Invitrogen). RNA quality was monitored by agarose gel electrophoresis, and RNA quantity was determined by spectrophotometry.

### 5’-RACE AND 3’-RACE

Following generation of cDNA sample from total RNA through reverse transcription (RT), 5’- or 3’-rapid amplification of cDNA ends was done using SMARTer RACE cDNA Amplification Kit. After agarose gel electrophoresis, the 5’-RACE or 3’-RACE fragment was recovered and purified with TaKaRa MiniBEST Agarose Gel DNA Extraction Kit, and sequenced with ABI-3700 automated DNA sequencer.

### PRIMER EXTENSION ASSAY

As described in our previous studies (Sun et al., 2012; Zhang et al., 2013a,b), a 5’-32P-labeled oligonucleotide primer complementary to a portion of the RNA transcript of each indicated gene was employed to synthesize cDNAs from total RNA templates using Promega Primer Extension System. If different *Y. pestis* strains were involved in a single experiment, equal amounts of total RNA...
were used as starting materials. Sequence ladders were prepared with the same 5′-32P-labeled primers using AccuPower & Top DNA Sequencing Kit (Bioneer). Radioactive species were detected with intensities of primer extension product. The size of primer extension product, while the relative mRNA levels (transcription start) of each target gene was mapped according to fluoride membranes (Immobilon P; Millipore), and incubated with the same 5′-32P-labeled primers using AccuPower & Top.

**LacZ FUSION AND β GALACTOSIDASE ASSAY**

A promoter-proximal DNA region of each indicated gene was cloned into the low-copy-number transcriptional fusion vector pRW50 (Lodge et al., 1992) that harbors a promoterless lacZ reporter gene. *Y. pestis* strains transformed with the recombinant plasmid or the empty pRW50 (negative control) were grown to measure β-galactosidase activity in cellular extract using β-Galactosidase Enzyme Assay System (Promega) (Sun et al., 2012; Zhang et al., 2013a,b).

**ANTIBODY PREPARATION AND WESTERN BLOT**

The 6× His-tagged peptide fragments of HmsT (a.a.285–390), HmsD (a.a.221–425), HmsF (a.a.193–482), and HmsP (a.a.441–671) were over-expressed, respectively, in BL21 (DE3) cells using pET28a vectors. Each recombinant protein was purified under denaturing conditions with Ni-NTA Agarose Column, and further prepared as soluble protein sample after renaturation for further immunization of New Zealand rabbits. The specific polyclonal IgG antibody was separated from rabbit serum by ammonium sulfate precipitation. For Western blot, cleared whole-cell lysate was prepared from harvested bacterial cells through sonication, followed by determination of protein concentrations with Bio-Rad protein assay kit. If different *Y. pestis* strains were involved in a single experiment, equal amounts of protein sample were separated on SDS-PAGE, immunoblotted to polyvinylidene fluoride membranes (Immobilon P; Millipore), and incubated with primary antibody and then goat anti-rabbit IRDye®800CW second antibody. Signals were detected with Odyssey Sa Infrared Imaging System.

**BIOFILM AND c-di-GMP ASSAYS**

As described in our previous study (Fang et al., 2013), three different methods were used to detect *Y. pestis* biofilms. First, *in vitro* biofilm masses, attached to well walls when bacteria were grown in polystyrene microtiter plates, were stained with crystal violet. Second, percentages of fourth-stage larvae and adults (L4/adult) of *C. elegans* after incubation of nematode eggs on *Y. pestis* lawns, negatively reflecting bacterial ability to produce biofilms, were determined. Third, rugose colony morphology of bacteria grown on LB agar plates, positively reflecting bacterial ability to synthesize exopolysaccharide, was observed. In addition, intracellular c-di-GMP levels were determined by a chromatography-coupled tandem mass spectrometry (HPLC-MS/MS) method as described in our previous study (Sun et al., 2012).

**EXPERIMENTAL REPLICATES AND STATISTICAL METHODS**

For LacZ fusion, crystal violet staining of biofilms, and determination of L4/adult nematodes or c-di-GMP, experiments were performed with at least three independent bacterial cultures/lawns, and values were expressed as mean ± standard deviation. Paired Student’s t-test was performed to determine statistically significant differences; *P* < 0.01 was considered to indicate statistical significance. For primer extension, Western blot, and colony morphology observation, representative data from at least two independent bacterial cultures were shown.

**RESULTS**

The 5′ and 3′ termini of HmsB were determined by 5′-RACE and 3′-RACE, respectively. The 262-bp hmsB gene, situated from nucleotide position 4,72,430–4,72,691 on *Y. pestis* CO92 genome, was located within the intergenic region of mgtA (YPO0451) and hmsC, and the two adjacent genes hmsB and hmsC were transcribed with opposite direction (Figure 1).

Determination of the growth curves of WT, ΔhmsB and c-hmsB showed that the hmsB deletion had no affect on bacterial growth *in vitro* (data not shown). Crystal violet could steadily stain *in vitro* biofilm masses produced by WT or c-hmsB; by contrast, ΔhmsB stained a great deal less crystal violet (Figure 2A), and as expected, almost no crystal violet staining could be detected for the reference biofilm-negative strain ΔhmsS. After incubation of nematode eggs on bacterial lawns of WT or c-hmsB, only a small portion (below 20%) of larvae grew and developed to L4/adult nematodes due to abundant attachment of *Y. pestis* biofilms on nematode heads; by contrast, bacterial lawns of ΔhmsB and ΔhmsS gave the percentage values of about 65 and 100%, respectively (Figure 2B). These indicated that the hmsB deletion compromised biofilm formation both *in vitro* and on nematodes. When grow on agar plates, WT and c-hmsB gave similar rugose colony morphology due to abundant biosynthesis of exopolysaccharide, ΔhmsS produced very smooth colonies, while ΔhmsB lied between ΔhmsS and WT/c-hmsB (Figure 2C).

Intracellular c-di-GMP concentrations were determined in WT, ΔhmsB, and c-hmsB by a HPL-MC/MS method. Compared to WT or c-hmsB, a significantly decreased production of c-di-GMP was observed for ΔhmsB (Figure 2D). As expected, almost no c-di-GMP could be detected for the reference c-di-GMP-negative strain ΔhmsTΔhmsD. In addition, there were similar observations of bacterial growth curve, c-di-GMP concentration, and crystal violet staining of biofilms in WT, ΔhmsB and c-hmsB (data not shown), when bacteria were grown in Brain Heart Infusion (BHI) broth or in chemically defined TMH medium (Straley and Bowmer, 1986). Taken together, the above results indicated that HmsB enhances c-di-GMP and exopolysaccharide production, which could account for HmsB-dependent lesion of biofilm formation.

ΔhmsT, hmsHFRS, hmsCDE, hmsP and its own gene were subjected to the following gene regulation assays for characterization of HmsB-dependent expression of these target genes. Levels of gene expression and protein biosynthesis were determined in WT and ΔhmsB but not the complemented mutant strain c-hmsB. This design was based on the following two observations: c-hmsB and WT gave very similar c-di-GMP and biofilm phenotypes (see above); and no change in expression of hmsC (upstream of hmsB) or mgtA (downstream) was detected in c-hmsB relative to WT by using quantitative RT-PCR and primer extension (data not shown).
FIGURE 2 | Involvement of HmsB in biofilm formation and c-di-GMP biosynthesis. (A) Crystal violet staining. Y. pestis was grown in 24-well polystyrene dishes, and the bacterial biomass (in vitro biofilms) attached to well walls were stained with crystal violet to determine OD$_{570}$ values. The planktonic cells were subjective to determine OD$_{620}$ values. The relative capacity of biofilm formation of each strain tested was shown with values of $500 \times$ OD$_{570}$/OD$_{620}$. (B) C. elegans biofilms. After incubation of nematode eggs on lawns of indicated Y. pestis strains, the developmental stages of nematodes on each lawn were scored to calculate percentage of L4/adult. (C) Bacterial colony morphology. Aliquots of bacterial glycerol stocks were spotted on LB plate, followed by incubation for one week. (D) Intracellular c-di-GMP concentration. The intracellular c-di-GMP concentrations were determined by a HPLC-MS/MS method, and the determining values were expressed as pmol/mg of bacterial protein.

The relative mRNA level of each of hmsB (Figure 3A), hmsC (Figure 4A), hmsT (Figure 5A), and hmsH (Figure 6A) was measured in WT or ΔhmsB by primer extension assay, and the results showed that the mRNA level of each of the above four genes decreased considerably in ΔhmsB relative to WT. Notably, this assay detected a single transcription start site (nucleotide A) located at nucleotide position 472430 on CO92 genome, which confirmed the above 5′-RACE result. The promoter-proximal region of each of hmsB (Figure 3B), hmsC (Figure 4B), hmsT (Figure 5B), and hmsH (Figure 6B) was cloned into the transcriptional lacZ fusion reporter vector pRW50, and the corresponding recombinant vector was introduced into WT or ΔhmsB to determine the target promoter activity; it was shown that the promoter activity of each of the above four genes was significantly reduced in ΔhmsB relative to WT. Further Western blot assay confirmed that biosynthesis of each of HmsD (Figure 4C), HmsT (Figure 5C), and HmsF (Figure 6C) decreased in ΔhmsB relative to WT. Notably, observations from transcriptional lacZ fusion experiments denoted that HmsB-dependent expression of hmsB, hmsCDE, hmsT, and hmsHFRS most likely involved mechanisms of gene transcriptional regulation.

By contrast, primer extension (Figure 7A) and Western blot (Figure 7B) assays indicated negative regulation of hmsP by HmsB at mRNA and protein levels, respectively. Further transcriptional lacZ fusion experiments (Figure 7C) indicated that HmsB had no regulatory effect on hmsP promoter activity.

DISCUSSION

Data presented here showed that Y. pestis sRNA HmsB enhanced the production of c-di-GMP, exopolysaccharide, and biofilm. In addition, HmsB stimulated expression of hmsB, hmsCDE, hmsT, and hmsHFRS, all of which encoded biofilm-enhancing factors,
Fang et al. HmsB enhances biofilm formation in *Yersinia pestis*

**FIGURE 3** | HmsB-dependent expression of *hmsB*. (A) Primer extension. The mRNA levels of *hmsB* in WT or Δ*hmsB* were determined by primer extension. The Sanger sequence ladders (lanes G, C, A, and T) and the primer extension products of *hmsB* were analyzed with an 8 M urea-6% acrylamide sequencing gel. The transcription start site of *hmsB* was indicated by underlined nucleotide A. (B) LacZ fusion. The PhmsB-lacZ transcriptional fusion vector was transformed into WT or Δ*hmsB*, and then the *hmsB* promoter activities (miller units of β-galactosidase activity) were determined in bacterial cellular extracts.

**FIGURE 4** | HmsB-dependent expression of *hmsCDE*. Primer extension (A) and LacZ fusion (B) experiments were done for *hmsC* as described in Figure 3. The 5’ terminus of RNA transcript (i.e., transcription start) of *hmsC* was indicated by arrow with nucleotide A, and the minus numbers under arrow indicated nucleotide position upstream of *hmsC*. For Western Blot (C), whole-cell protein extract from WT or Δ*hmsB* or Δ*hmsD* (negative control) was loaded for SDS-PAGE and incubated with anti-HmsD antibodies. Noted that the first gene of *hmsCED* was subjected to gene expression assays (A,B), while diguanylate cyclase HmsD was chosen for protein biosynthesis analysis (C).

while repressed that of *hmsP* encoding a biofilm-inhibiting factor. HmsB appeared to act as a major activator of biofilm formation in *Y. pestis*. To the best of our knowledge, this is the first report of a sRNA regulating *Yersinia* biofilm formation.

HmsB had regulatory effect on promoter activity of *hmsB*, *hmsCDE*, *hmsT*, and *hmsHFRS* but not that of *hmsP*. Commonly, sRNAs inhibit the translation of their mRNA targets by base pairing with the neighborhoods of ribosomal binding sites (RBSs) to
block ribosome binding and thus to inhibit protein biosynthesis (Han et al., 2013). Less commonly in cases studied to date, sRNAs can activate translation by freeing RBSs that would otherwise be occluded by inhibitory secondary structures (Han et al., 2013). Whether HmsB binds to RBS-around regions of these hms genes needs to be elucidated.

The positive regulatory action of HmsB on the promoter activity of hmsB, hmsCDE, hmsT, and hmsHFRS, as characterized in this work, are highly unusual; it is speculated that HmsB modulates the translation of one or more transcriptional activators or repressors of the above hms genes. It should be noted that multiple transcriptional regulators of Y. pestis biofilm formation have
be identified (Sun et al., 2012; Rebeil et al., 2013; Tam et al., 2014). The hfq deletion led to dramatic degeneration of HmsB in Y. pestis (unpublished data). Most of sRNAs characterized to date need binding of Hfq as a RNA chaperone, stabilizing formation of imperfect sRNA-target RNA duplexes (Han et al., 2013). It has been characterized that Hfq is essential for the biofilm formation and flea blockage of Y. pestis strain KIM6+ during colonization of flea gut (Rempe et al., 2012). Positive control of biofilm formation by Hfq is also observed in Y. pestis strain 201 used in this study, and further gene regulation assays show that Hfq enhances the expression of hmsCDE, hmsT, and hmsHFRS but inhibits that of hmsP in this strain (unpublished data). By contrast, a separate study reports that Hfq is a repressor of biofilm formation through inhibiting expression of hmsCDE, hmsT, and hmsHFRS but stimulating that of hmsP in a pCD1- derivative of Y. pestis CO92 (Bellows et al., 2012); interestingly, similar results can be observed in Y. pestis strain 201 cured of pCD1 (unpublished data).

The sRNA Ysr141 targets an untranslated region upstream of yopJ to posttranscriptionally activate synthesis of YopJ, an effector protein of the Yop-Ysc type III secretion system (Schiano et al., 2014); this is up to now the only report of sRNA-target gene association in Y. pestis. Although HmsB-dependent expression hms genes have been dissected in the present work, direct HmsB targets (probably including not only hms genes but their upstream transcriptional regulators) as well as detailed mechanisms of action of HmsB in aid of Hfq on its target genes needs to be dissected to understand how HmsB contributes to biofilm gene regulation.

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SUPPLEMENTARY MATERIAL

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