RcsAB is a major repressor of *Yersinia* biofilm development through directly acting on hmsCDE, hmsT, and hmsHFRS

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Biofilm formation in flea gut is important for flea-borne transmission of *Yersinia pestis*. There are enhancing factors (HmsHFRS, HmsCDE, and HmsT) and inhibiting one (HmsP) for *Yersinia pestis* biofilm formation. The RcsAB regulatory complex acts as a repressor of *Yersinia* biofilm formation, and adaptive pseudogenization of *rcsA* promotes *Y. pestis* to evolve the ability of biofilm formation in fleas. In this study, we constructed a set of isogenic strains of *Y. pestis* biovar *Microtus*, namely WT (*RscB*+ and *RcsA*−), c-rcsA (*RscB*+ and *RcsA*+), *ArcsB* (*RscB*− and *RcsA*−), and *ArcsB*/c-rcsA (*RscB*− and *RcsA*+). The phenotypic assays confirmed that *RcsB* alone (but not *RcsA* alone) had an inhibiting effect on biofilm/c-di-GMP production whereas assistance of *RcsA* to *RcsB* greatly enhanced this inhibiting effect. Further gene regulation experiments showed that *RcsB* in assistance of *RcsA* tightly bound to corresponding promoter-proximal regions to achieve transcriptional repression of hmsCDE, hmsT and hmsHFRS and, meanwhile, *RcsAB* positively regulated hmsP most likely in an indirect manner. Data presented here disclose that pseudogenization of rcsA leads to dramatic remodeling of RcsAB-dependent hms gene expression between *Y. pestis* and its progenitor *Y. pseudotuberculosis*, enabling potent production of *Y. pestis* biofilms in fleas.

*Yersinia pestis* is an extremely virulent pathogen causing severe invasive infections mainly manifested as bubonic plague in lymph nodes, septicemic plague in blood vessels, and pneumatic plague in lungs. *Y. pestis* is potent to synthesize biofilms, which are a population of bacterial colonies embedded in self-produced matrix. Formation of attached *Y. pestis* biofilms in flea gut is important for flea-borne transmission of this pathogen. *Y. pestis* biofilm matrix is primarily composed of poly-B-1,6-N-acetylglucosamine exopolysaccharide. The hmsHFRS operon is responsible for biosynthesis and translocation of biofilm exopolysaccharide through cell envelope. HmsR and HmsS are located in inner membrane whereas HmsH and HmsF are outer-membrane proteins. HmsR has four transmembrane domains plus a cytoplasmic glycosyltransferase domain, while HmsS has two transmembrane domains; HmsR and HmsS form an enzymatic complex responsible for exopolysaccharide biosynthesis. HmsH acts as a porin with β-barrel structure, and HmsF functions as a polysaccharide deacetylase; these two proteins form a complex for modification/export of partially deacetylated exopolysaccharide through outer membrane.

The 3′,5′-cyclic diguanosine monophosphate (c-di-GMP), a small-molecule second messenger promoting exopolysaccharide biosynthesis, is produced from guanosine triphosphate by GGDEF-domain-containing diguanylate cyclases and degraded by EAL-domain-containing phosphodiesterases. *Y. pestis* produces a total of two diguanylate cyclases HmsT and HmsD, and both of them are required for c-di-GMP biosynthesis and biofilm formation. Although expression of both HmsT and HmsD is up-regulated in flea gut and upon temperature shift from 37°C (as in warm-blooded hosts) to 26°C (in flea gut), HmsD plays a major role in biofilm formation in fleas while the predominant effect of HmsT is on *in vitro* biofilm formation. The hmsD gene is a member of the three-gene operon hmsCDE. HmsD is a trans-inner-membrane protein composed of three distinct domains, namely a periplasmic sensor domain, an HAMP signal converter domain and a cytoplasmic output GGDEF domain. The periplasmic protein HmsC senses environmental signals and then interacts with HmsD periplasmic domain, which affects HmsD stability and thereby regulates cellular c-di-GMP levels. In addition, *Y. pestis* expresses the sole c-di-GMP-specific phosphodiesterase HmsP, which is responsible for degradation of c-di-GMP and therefore has an inhibiting effect on biofilm formation.
The hmsHFRS orthologs can be found in several bacterial species, including the genetically close pgaABCD operon in Escherichia coli. c-di-GMP binds to PgaC and PgaD (homologues of HmsR and HmsS, respectively), which stabilizes the PgaCD enzymatic complex and thereby activates its glycosyltransferase activity to produce exopolysaccharide. Without c-di-GMP binding, PgaD fails to interact with PgaC and both of them are subject to proteolysis. Y. pestis might employ the conserved c-di-GMP-HmsRS association mechanism to control exopolysaccharide production.

The Enterobacteriaceae Rcs phosphorelay system is an atypical two-component regulatory system composed of three proteins, RcsB, RcsC and RcsD. RcsC and RcsD are membrane-bound proteins, while RcsB is a cytoplasmic one. RcsC acts as a sensor kinase catalyzing autophosphorylation of RcsD and RcsB, and the resulting phosphate group is then transferred to RcsD and finally to RcsB. Phosphorylated RcsB (RcsB-p) acts as a transcriptional repressor. The RcsAB complex recognizes a consensus box sequence TAAGAAT-ATTCTA, which is a 7-7 invert repeat, within the promoter-proximal regions of its target genes mainly including those responsible for exopolysaccharide biosynthesis, flagellar mobility, and Rcs autoregulation (Table S1, and Fig S1).

The biofilm formation of Y. pestis and its genetically very closed progenitor Y. pseudotuberculosis is negatively regulated by the Rcs phosphorelay system. The rcsA gene is inactivated in Y. pestis due to a 30 bp duplication insertion in its coding region, and replacing the rcsA pseudogene with functional rcsA allele of Y. pseudotuberculosis strongly represses Y. pestis biofilm formation and essentially abolishes flea blockage. The conversion of rcsA to a pseudogene during evolution from Y. pseudotuberculosis to Y. pestis is most likely a case of positive Darwinian selection.

The present work discloses that the RcsAB complex acts as a major repressor of Y. pestis biofilm formation through directly repressing transcription of hmsCDE, hmsT and hmsHFRS meanwhile positively regulating hmsP in an indirect manner. The above results denote dramatic remodeling of biofilm-related hms gene expression between Y. pestis and its progenitor Y. pseudotuberculosis due to adaptive pseudogenization of a regulatory gene rcsA.

**Results**

**Bacterial strains and their biofilm phenotypes.** Transformation of pACYC184-rcsA into WT (wild-type, RscB+ and RcsA-) generated the rcsA-complemented strain c-rcsA (RscB+ and RcsA+), which
Figure 2 | RcsAB-dependent expression of hmsCDE. (a) Primer extension. The relative mRNA levels of hmsC in indicated strains were determined by primer extension. The Sanger sequence ladders (lanes G, C, A, and T) and the primer extension products of hmsC were analyzed with an 8 M urea-6% acrylamide sequencing gel. The transcription start site of hmsC was indicated by arrow with nucleotide A, and the minus number under arrow indicated the nucleotide position upstream of hmsC start codon. (b) LacZ fusion. The hmsC::lacZ transcriptional fusion vector was transformed into indicated strains, and then hmsC promoter activities (miller units of β-galactosidase activity) were determined in bacterial cellular extracts. (c) EMSA. The radioactively labeled DNA fragments were incubated with indicated purified proteins and then subjected to a native 4% polyacrylamide gel electrophoresis. (d) DNase I footprinting. Labeled coding or non-coding DNA probes were incubated with indicated purified proteins and then subjected to DNase I digestion. The footprint regions were indicated with vertical bars. Lanes C, T, A, and G represented Sanger sequencing reactions. The DNA-binding of His-RcsB-p in presence of MBP-RcsA (involved in EMSA and DNase I footprinting) and that of His-RcsB-p alone (in EMSA) were tested. (d) Promoter structure. Shown were with translation/transcription starts, core promoter −10 and −35 elements, SD sequences, RcsAB sites, and RcsAB box-like sequences.
led to a considerable decrease in c-di-GMP/biofilm production (Fig. 1).

Deletion of rcsB from WT generated the rcsB-null strain ΔrcsB (RscB- and RcsA-), and further transformation of pACYC184-rcsA into ΔrcsB generated another rcsA-complemented strain ΔrcsB/c-rcsA (RscB- and RcsA+); compared to WT, both ΔrcsB and ΔrcsB/c-rcsA gave significantly enhanced c-di-GMP/biofilm production (Fig. 1).

Transformation of pACYC184-rcsB into ΔrcsB generated the rcsB-complemented strain c-rcsB (RscB+ and RcsA-), which had a biofilm/c-di-GMP production phenotype very similar to WT (Fig. 1).

Taken together, RcsB alone (but not RcsA alone) has an inhibiting effect on biofilm/c-di-GMP production, whereas assistance of RcsA to RcsB greatly enhances this inhibiting effect.

**Regulation of hmsCDE, hmsT, hmsHFRS and hmsP by RcsAB**

RcsAB box-like sequences could be found within the promoter-proximal regions of hmsCDE, hmsT and hmsHFRS, indicating that they might serve as direct RcsAB targets (Table S3), which promoted us to elucidate RcsAB-dependent expression of these candidate genes. hmsP was also included in the following gene regulation analyses.

The primer extension assays indicated the relative mRNA levels of each of hmsC (Fig. 2a), hmsT (Fig. 3a) and hmsH (Fig. 4a) in the below four strains showed the following tendency: c-rcsA < WT < ΔrcsB = ΔrcsB/c-rcsA. This observation was further confirmed by determination of the promoter activities of the above four genes by LacZ fusion (Fig. 2b, Fig. 3b, and Fig. 4b). As determined by electrophoretic mobility shift assay (EMSA), His-RcsB-p alone or mixed with excess MBP-RcsA could bind to the promoter-proximal region of each of hmsC (Fig. 2c), hmsT (Fig. 3c) and hmsH (Fig. 4c) in a dose-dependent manner; moreover, addition of excess RcsA could improve DNA-binding activity of RcsB-p. Further DNase I footprinting experiments showed that His-RcsB-p in presence of...
MBP-RcsA protected a single upstream region of each of hmsC (Fig. 2d), hmsT (Fig. 3d) and hmsH (Fig. 4d). The above observations indicated that RcsB-p in assistance of RcsA tightly bound to the corresponding promoter-proximal regions to achieve transcriptional repression of hmsCDE, hmsT and hmsHFRS.

By contrast, the relative mRNA levels (determined by primer extension, Fig. 5a) of hmsP showed the following tendency: c-rcsA > WT > ΔrcsB ≈ ΔrcsB/c-rcsA, which was further validated by quantitative RT-PCR (data not shown). However, LacZ fusion assay (Fig. 5b) indicated that RcsAB had no regulatory effect on hmsHFRS.

Figure 4 | RcsAB-dependent expression of hmsHFRS. Primer extension (a), LacZ fusion (b), EMSA (c), and DNase I footprinting (d) experiments were performed as described in Fig 2.
promoter activity of hmsP. In addition, both EMSA (Fig. 5c) and DNase I footprinting (data not shown) indicated no association between RcsAB and hmsP upstream DNA. Therefore, RcsAB positively regulated hmsP most likely in an indirect manner.

Organization of RcsAB-dependent promoters. Transcription starts determined by primer extension were considered as transcribed promoters for indicated genes and, accordingly, core promoter 210 and 235 elements could be predicted. Each of hmsCD (Fig. 2e), hmsT (Fig. 3e), hmsP (Fig. 4e) and hmsHFRS (Fig. 5d) had a single transcribed promoter. It should be noted that all the above data were consistent with our previous report on regulation of hms genes by Y. pestis ferric uptake regulator Fur20.

The footprints determined by DNase I footprinting were considered as RcsAB sites for hmsCDE, hmsT, and hmsH; a expected, RcsAB box-like sequences (Table S3) could be found within all these RcsAB sites. The organization of RcsAB-dependent promoters of hmsCDE (Fig. 2e), hmsT (Fig. 3e), hmsHFRS (Fig. 4e), and hmsP (Fig. 5d) was constructed with translation/transcription starts, core promoter –10 and –35 elements, predicted Shine-Dalgarno (SD) sequences for ribosomal binding, RcsAB sites, and RcsAB box-like sequences.

Discussion

Transcriptional repression of genes for biofilm exopolysaccharide biosynthesis by RcsB with assistance of its auxiliary protein RcsA has been characterized in several bacterial species (Table S1). The present work confirms RcsAB-mediated tight inhibition of Y. pestis c-di-GMP/exopolysaccharide/biofilm production by using a set of isogenic strains of Y. pestis biovar Microtus, namely WT (RscB+ and RcsA+), c-rcsA (RscB+ and RcsA+), ArcsB (RscB- and RcsA+), and ArcsB/-rcsA (RscB- and RcsA+). RcsAB acts as a major repressor of Y. pestis biofilm formation through directly repressing transcription of biofilm-enhancing genes hmsCDE, hmsT and hmsHFRS and meanwhile positively regulating biofilm-enhancing one hmsP in an indirect manner. RcsB in absence of RcsA does have residual regulatory effects on biofilm formation and hms gene expression and, moreover, RcsB-dependent regulation is greatly increased with assistance of RcsA, which was consistent with previous results19,21,22. The above regulatory circuit leads to different expression levels of each of hmsCDE, hmsT, hmsHFRS and hmsP in the above isogenic strains and thus distinct potencies of these strains to produce c-di-GMP/biofilm (summarized in Fig. 6).

Y. pseudotuberculosis (RscB+ and RcsA+, analogous to Y. pestis strain c-rcsA in this study) has a biofilm phenotype in fleas19,21,22. In Y. pseudotuberculosis, biosynthesis of HmsCDE, HmsT, and HmsHFRS is tightly inhibited while HmsP is allowed to express. The pseudogenization of rcsA leads to inability of RcsAB complex in Y. pestis, which in turn alleviates RcsAB-mediated inhibition of expression of hmsCDE, hmsT, and hmsHFRS. As a prerequisite of potent Y. pestis biofilm formation, the adaptive pseudogenization of rcsA results in dramatic remodeling of hms gene expression patterns between Y. pseudotuberculosis and Y. pestis, finally enabling Y. pestis biofilm formation in fleas and thereby flea-borne transmission of this pathogen.
Table 1 | Y. pestis strains involved in gene deletion and complementation

| Strain     | rcsA | rcsB | hmsD | hmsT | hmsS |
|------------|------|------|------|------|------|
| WT         | +    | -    | +    | +    | +    |
| c-rcsA     | -    | +    | +    | +    | +    |
| ΔrcsB      | -    | +    | +    | +    | +    |
| ΔrcsB/ΔrcsA| +    | -    | +    | +    | +    |
| ΔhmsTΔhmsD | -    | +    | -    | +    | +    |

| Feature                  | Reference          |
|--------------------------|--------------------|
| The wild-type Y. pestis biovar Microtus strain 201. | [27] |
| The vector pACYC184-rcsA was introduced into WT. | This study |
| The base pairs 211 to 418 of rcsB gene was deleted from WT. | This study |
| The vector pACYC184-rcsB was introduced into ΔrcsB. | This study |
| A reference c-di-GMP strain. The base pairs 4 to 1179 of hmsT gene was deleted from WT, and then the base pairs 41 to 1238 of hmsD gene was deleted from ΔhmsT. | This study |
| A reference biofilm strain. The base pairs 146 to 468 of hmsS was deleted from WT. | [20] |

Methods

Bacterial strains. The wild-type Y. pestis Microtus strain 201 (WT) is avirulent to humans but highly virulent to mice [27]. The partial coding region of each indicated gene was replaced by a kanamycin resistance cassette by using the one-step inactivation method based on the lambda phage recombination system [26], to generate the corresponding 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 pACYC184 (GenBank accession no. X06403), and the resulting recombinant vector was transformed into each indicated Y. pestis strain lack of the corresponding functional gene, generating the corresponding complemented mutant (Table 1). All the primers designed in this study are listed in Table S2.

Bacterial growth and RNA isolation. Overnight cell cultures in the Luria-Bertani (LB) broth with an optical density (OD_{600}) 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 middle stationary phases (an OD_{600} of 0.8 to 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 mixed with 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.

Primer extension assay. As described in our previous studies [23,29], a 5′-32P-labeled oligonucleotide primer complementary to a portion of the RNA transcript of each indicated gene was employed to synthesize cdNA from total RNA templates using Promega Primer Extension System. Sequence ladders were prepared with the same 5′-32P-labeled primers using AccuPower & Top DNA Sequencing Kit (Bioneer). Radiolabeled primers were separated by electrophoresis in a denaturing gel, and radioactive products were detected by autoradiography.

Protein expression and purification. The entire coding region of Y. pseudotuberculosis rcsA or Y. pestis rcsB was cloned into plasmid pMAL-c4X (Invitrogen) or pBADMyc-His A (New England Biolabs), respectively. The wild-type Y. pestis strain KIM6+ and the rcsB null mutant of KIM6+ were employed as host cells for expression of maltose-binding protein (MBP)-tagged RcsA (MBP-RcsA) and 6×His-tagged RcsB (His-RcsB), respectively [23]. His-RcsB and MBP-RcsA were purified under native conditions using Ni-NTA Arose Column (Qiagen) and Amylose Arose Column (New England Biolabs), respectively [23]. Each purified protein was dialyzed and then concentrated to a concentration of about 0.1 mg/ml in phosphate buffered saline (pH 8.0) containing 20% glycerin.

EMSA. Each indicated 5′-32P-labeled target DNA fragment was incubated with increasing amounts of purified His-RcsB, or with increasing amounts of purified His-RcsB with addition of 24 pmol of purified MBP-RcsA, for 30 min at room temperature in a binding buffer [23]. To achieve RcsB phosphorylation, 25 mM fresh acetyl phosphate was incubated for 30 min with His-RcsB in the binding buffer.
before labeled DNA probes were added. The resulting reactions were subjected to a native 4% (w/v) polyacrylamide gel electrophoresis. Each EMSA experiment included three controls, namely, cold probe as the specific DNA competitor (the same promoter–proximal DNA region labeled), negative probe as the nonspecific DNA competitor (the unlabeled coding region of the 16S rRNA gene), and nonspecific protein competitor (rabbit anti-Fli protein polyclonal antibodies)29-30. Detection of sequencing and radioactive species was as above.

DNase I footprinting. For DNase I footprinting29-30, the target DNA fragment with a single 32P-labeled end was incubated with increasing amounts of purified His-RcsB-p with addition of 24 pmol of purified MBP-RcsA, which was followed by partial digestion of Bqi RNase-Free DNase I (Promega). The digested DNA samples were purified and analyzed in an 8 M urea-6% polyacrylamide gel. Detection of sequencing and radioactive species was as above. Footprints were identified by comparison with sequence ladders.

Biofilm and c-di-GMP assays. As described in our previous study8, 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 produce biofilms, were determined. 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 study29.

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 and colony morphology observation, shown were representative data from at least two independent bacterial cultures.

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Author contributions
D.Z. and R.Y. designed experiments. N.F., H.Y., H.F., L.L, Y.Z., L.W., Y.H., D.Z. and R.Y. contributed reagents, materials and analysis tools. D.Z. and R.Y. wrote this manuscript.

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