Differential regulation of the hmsCDE operon in Yersinia pestis and Yersinia pseudotuberculosis by the Rcs phosphorelay system

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Yersinia pestis, the agent of plague, forms a biofilm in its flea vector to enhance transmission. Y. pestis biofilm development is positively regulated by hmsT and hmsD, encoding diguanylate cyclases (DGCs) involved in synthesis of the bacterial second messenger c-di-GMP. rcsA, encoding an auxiliary protein in Rcs phosphorelay, is nonfunctional in Y. pestis, while in Yersinia pseudotuberculosis, rcsA is functional and represses biofilms. Previously we showed that Rcs phosphorelay negatively regulates transcription of hmsT in Y. pestis and its ancestor Yersinia pseudotuberculosis. In this study, we show that Rcs positively regulates hmsCDE operon (encoding HmsD) in Y. pestis; while in the presence of functional rcsA, Rcs represses hmsCDE operon in Y. pseudotuberculosis. Loss of rcsA’s function in Y. pestis not only causes derepression of hmsT but also causes activation of hmsD, which may account for the increased biofilm formation in Y. pestis. In addition, differential regulation of the two DGCs, HmsT and HmsD by Rcs may help Y. pestis to adapt to different environment.
Results

Functional RcsA represses the hmsCDE operon. We previously reported that Y. pestis rcsA is mutated to a nonfunctional pseudogene and that introducing the functional Y. pseudotuberculosis rcsA into Y. pestis results in decreased biofilm formation20. Further study showed that Rcs phosphorylated repressed transcription of the diguanylate cyclase gene hmsT22. However, Y. pestis with functional rcsA forms almost no biofilm in the digestive tract of fleas, whereas the Y. pestis hmsT mutant forms intermediate levels of biofilm in the digestive tract of fleas24, suggesting that rcsA may regulate other target genes to repress Yersinia spp. biofilms.

Since hmsD, another DGC encoding gene in Y. pestis, plays a more important role in the biofilm formation in vivo24, we hypothesized that hmsD may be regulated by Rcs. To verify this hypothesis, we constructed transcriptional fusions using E. coli lacZ as the reporter. As hmsD is located in the hmsCDE operon, hmsC::lacZ was constructed in the pGD926 vector26. hmsT::lacZ and lcrQ::lacZ were constructed in the pGD926 vector as positive and control vectors. The resulting plasmids were transformed into Y. pestis strains, and we found that RcsB activated the expression of HmsD in wild type, Figure 1c, lane 3. Taken together, these results suggest functional rcsA represses the transcription of hmsCDE in Y. pestis.

RcsB positively regulates the hmsCDE operon. We next used the lacZ reporters to determine the effects of RcsB. To our surprise, over-expression of RcsB produced a moderate increase while mutation of RcsB resulted in a slight decrease of the promoter activity of hmsC (Figure 1a). The positive control hmsT::lacZ was repressed while the negative control lcrQ::lacZ was not affected by over-expression of RcsB (Supplementary Fig. S1). qRT-PCR and western analysis confirmed that deletion of RcsB resulted in decreased expression of hmsD, while over-expression of RcsB activated the expression of hmsD (Figure 1b and c). Phosphorylation of aspartic acid 56 (D56) is usually required for RcsB’s function. Reversion D56S with glutamic acid (E) can mimic the phosphorylated status of RcsB, while mutation of D56 with glutamine (Q) abolishes the phosphorylation of RcsB22,23,24. Over-express of RcsB (D56E) but not RcsB(D56Q) results in induction of hmsC expression (Figure 1a), suggesting that phosphorylation of RcsB is necessary for the regulation of hmsC. Consistent with this and the previous finding that RcsD might dephosphorylate RcsB in Y. pestis22,23, mutation of rcsD resulted in slightly increased expression of HmsD (11% of the levels of wild type, Figure 1c), while over-expression of RcsD produced a moderate decrease in expression of HmsD (61% of the levels of wild type, Figure 1c). These results imply that RcsB positively regulates the hmsCDE operon.

Since hmsD is located in the hmsCDE operon and since hmsC negatively regulates the function of hmsD19, we wanted to know the effect of expression of the hmsCDE operon on biofilm formation. To detect this, we cloned the hmsCDE genes into the pVTRA vector, where the hmsCDE genes are driven by an IPTG-inducible promoter. The resulting plasmid was transformed into the Y. pestis hmsCDE mutant and analyzed for biofilm formation. Consistent with previous findings that deletion of hmsCDE operon resulted in decreased biofilm formation, over-expression of hmsCDE following addition of IPTG resulted in increased biofilm formation (Figure 2), suggesting that increased transcription of hmsCDE activates biofilm formation. Taken together, these results suggest that Rcs might control biofilm formation in Y. pestis by upregulating the hmsCDE operon.

An RcsAAB box in the hmsC promoter mediates the regulation of the hmsCDE operon by Rcs. Using two different 5’ RACE Kits (Methods), we determined the hmsCDE transcription start site to be 64 bp upstream of the initial ATG of hmsC. To verify this, we constructed a lacZ reporter with the mutated putative −10 box. The mutation of the −10 box resulted in nearly complete loss of activity of the hmsC promoter, indicating that the identified transcription start site is the sole or predominant one (Supplementary Fig. S2). To further rule out the possibility that hmsD has another promoter in the ORF of hmsC, we constructed a lacZ reporter that contained the hmsC ORF region but not its upstream sequence, and assayed for β-galactosidase activity. As shown in Supplementary Fig. S2, almost
no promoter activity was detected in the analysis. Taken together, these results suggested that 64-bp upstream of ATG of hmsC is the sole or predominant transcription start site.

An RcsAAB box is located within 59-bp upstream of the hmsC transcription start site, which matches the consensus sequence at 9 of 14 nucleotides, including all five of the most conserved nucleotides (Figure 3). We noticed that the left half of the conserved RcsAAB box is repeated immediately upstream of the RcsAB box. Since the right half of the RcsAAB box is believed to be the RcsB binding site,

To test the role of this region, we constructed a series of mutations of the RcsAAB box in the hmsC::lacZ fusion reporter (Figure 3) and analyzed the effects of Rcs on these mutated reporters in Y. pestis.

**hmsD promoter binding by RcsB.** We directly assayed RcsB binding to the RcsAAB box using an electrophoretic mobility shift assay (EMSA). The DNA probe was a 112-bp promoter sequence comprising the RcsAAB box, the putative RcsB binding site, and 2400 ng of RcsB was required to retard half of the probe, and 4000 ng for essentially complete shifting (Figure 6b, c, lanes 6, 8), which is similar to that of the negative control lcrQ promoter (Supplementary Fig. S3b). However, with the mutated RcsXXX or RcsAXX, more than 2000 ng of RcsB was required to retard half of the probe, and 4000 ng for essentially complete shifting (Figure 6a, lane 6). In contrast, more than 2000 ng of RcsB was required to retard half of the lcrQ probe, and 4000 ng for essentially complete shifting (Supplementary Fig. S3b). To further examine the role of RcsAAB box, we also performed EMSA for RcsB with the mutated HmsD promoter. The promoter DNA of hmsT and lcrQ were analyzed by EMSA as positive and negative controls. Similar to hmsT promoter (Supplementary Fig. S3a), half of the hmsD probe was retarded by 800 ng of RcsB (Figure 6a, lane 3), and at 2000 ng the free probe was almost undetectable (Figure 6a, lane 6).

**Discussion**

Y. pestis diverged from Y. pseudotuberculosis only 1,500-6,400 years ago. We previously obtained evidence suggesting that, during the evolution of Y. pestis, mutation of rcsA was the product of natural
selection and not genetic drift\textsuperscript{26}. The mutation was required for \textit{Y. pestis} to colonize its flea vector with a biofilm\textsuperscript{28,29}. We also previously found that a target of Rcs regulation is \textit{hmsT}\textsuperscript{24}, encoding a DGC that regulates biofilms. Repressing \textit{hmsT} apparently is not the only mechanism by which Rcs negatively regulates biofilms, because \textit{Y. pestis} \textit{hmsT} mutation and \textit{Y. pestis rcsA-pstB} strains have similar biofilm phenotype \textit{in vitro}, but different biofilm phenotypes \textit{in vivo}\textsuperscript{10,24}.

In this study, we showed that \textit{hmsCDE}, encoding another DGC (\textit{hmsD}) that regulates biofilms in \textit{Yersinia}\textsuperscript{28,31}, is also a target of Rcs regulation. Most importantly, we found a dual effect of the Rcs phosphorelay on expression of \textit{hmsD}: RcsA\textit{balona} activates \textit{hmsD} transcription, but RcsAB represses \textit{hmsD} transcription. Several lines of evidence support this conclusion. First, \textit{hmsD} transcription is reduced when functional RcsA is present (Figure 1a, b), and \textit{HmsD} protein levels are also reduced (Figure 1c and Supplementary Fig. S4a, lane 2). Secondly, \textit{hmsD} transcription is reduced when RcsB is mutated and stimulated when RcsB is over-expressed (Figure 1a), and \textit{HmsD} protein levels are consistent with the transcriptional levels (Figure 1c). Thirdly, deletion of \textit{hmsCDE} operon results in less biofilm formation but over-expression of \textit{hmsCDE} activates biofilm formation (Figure 2). Finally, we showed that RcsB binds to the \textit{hmsD} promoter in an RcsB binding site-dependent manner (Figure 6). Contrary to the finding that \textit{rcsB} positively regulates \textit{hmsCDE}, our previously result revealed that mutation of \textit{rcsB} resulted in increased biofilm formation \textit{in vitro}\textsuperscript{24,28}. It can be explained by the fact that \textit{hmsT} but not \textit{hmsD} plays a dominant role on \textit{in vitro} biofilm formation\textsuperscript{10}. Mutation of \textit{rcsB} causes increased expression of \textit{hmsT}\textsuperscript{24}, which may account for the increased biofilm formation \textit{in vitro}.

The Rcs phosphorelay signal transduction system is an important signaling pathway in the Enterobacteriaceae. It has been reported that RcsB functioned as an activator and also a repressor in the regulation of \textit{gadA} in \textit{E. coli}\textsuperscript{23}. RcsB/GadE heterodimer binds to the GAD box, while RcsB alone binds to the RcsB box. Compared with the affinity of the RcsB/GadE for the GAD box, the affinity of RcsB for RcsB box is very low\textsuperscript{23}. Thus, RcsB functions as an activator at lower concentration and as a repressor at high concentration in the regulation of \textit{gadA}. Apparently it is not the same case for the regulation \textit{hmsCDE}. An Rcs box is present at 59-bp upstream of the transcription start site of \textit{hmsCDE}. This box is necessary for the activation but not the repression of \textit{hmsCDE} transcription (Figure 4 and Figure 5). RcsB might bind to this box and then directly activate the transcription of \textit{hmsCDE}.

The regulatory mechanism by which RcsAB represses \textit{hmsCDE} transcription is still unknown. One hypothesis is that RcsAB acts

![Figure 4](https://www.nature.com/scientificreports/4)

**Figure 4 | Role of the RcsAAB box on transcriptional regulation of \textit{hmsCDE} by Rcs.** \(\beta\)-galactosidase activities of \textit{hmsC::lacZ} reporters with the RcsAAB box mutated to (a) RcsXXX, (b) RcsAXX, (c) RcsABX or (d) RcsXAB were analyzed. The mean and standard deviation of three independent experiments with three replicates are indicated. The mean and standard deviation of three independent experiments with three replicates are indicated. *\(P < 0.05\), **\(P < 0.01\).
indirectly by activating or repressing another regulator, which subsequently regulates hmsCDE. This hypothesis is supported by the facts that both RcsB and RcsAB negatively regulate hmsCDE expression when RcsAAB box is mutated to RcsXXX and RcsAXX (Figure 4 and Figure 5). In addition, there is still another question: why Y. pestis and Y. pseudotuberculosis have an RcsAAB box rather than an RcsAB box? One explanation is that the presence of left RcsA binding site affects the activation role of Rcs on hmsCDE. This hypothesis is supported by the result that Rcs regulation of hmsCDE is almost gone in the RcsXAB background (Figure 4d and Figure 5d).

Figure 5 | Role of the RcsAAB box on regulation of expression of HmsD by Rcs in Y. pestis. Western blots of total protein-matched lysates prepared from cells with mutation of (a) RcsXXX, (b) RcsAXX, (c) RcsABX or (d) RcsXAB were analyzed by anti-Myc antibody. Levels of HmsD were quantitated by using ImageJ from at least two independent experiments; numbers below blots indicate the ratio of HmsD from the indicated strain compared to that from wild-type hmsD-Myc2 strain (WT). Strain designations (Supplementary Table S1) are: 1, KIM6+; 2, hmsD-Myc2; 3, Rcs box mutation, hmsD-Myc2; 4, functional rcsA, Rcs box mutation, hmsD-Myc2; 5, ΔrcsB, Rcs box mutation, hmsD-Myc2; 6, ΔrcsB, Rcs box mutation, hmsD-Myc2/pUC19; 7, ΔrcsB, Rcs box mutation, hmsD-Myc2/pYC332; p-rcsB. Full-length blots are presented in Supplementary Fig. S6–S9 online.

Figure 6 | RcsB binds to the hmsC promoter. Electrophoretic mobility shift assays (EMSA) of hmsC promoter DNA incubated with increasing concentrations of RcsB. hmsD promoters with wild-type RcsAAB box (a) or with the mutated RcsAAB box RcsXXX (b), RcsAXX (c), RcsABX (d), or RcsXAB (e) were tested with identical protein combinations. Lane 1, hmsD probe alone; lanes 2–10, 100 ng hmsCDE probe with 400, 800, 1200, 1600, 2000, 2400, 4000, 6000 or 8000 ng of RcsB in the 16 µL reaction.

Our data support a model of multi-level control of hmsCDE by Rcs in Yersinia (Figure 7). In the presence of rcsB and functional rcsA, as is found in Y. pseudotuberculosis, RcsB together with RcsA repress the transcription of hmsCDE in an RcsB binding site-dependent manner. As a whole, Rcs negatively regulates hmsCDE expression in Y. pseudotuberculosis. In the presence of rcsB alone, as found in Y. pestis, rcsB directly activates and indirectly represses hmsCDE expression. As a whole, Rcs negatively regulates hmsCDE expression in Y. pestis. In Y. pseudotuberculosis, hmsT and hmsD transcriptions are tightly repressed. This tight repression was relieved during Y. pestis evolution by the mutation of rcsA to a pseudogene28. There remains a
residual repression of hmsT mediated by RcsB, but expression of hmsD is activated by RcsB. We know that HmsT plays a dominant role on in vitro biofilm formation, while HmsD plays a major role on biofilm formation in the flea. Thus RcsB negatively regulates Y. pestis biofilm formation in vitro, but might positively regulate biofilms in vivo. In summary, it appears that Y. pestis evolved to occupy the flea niche not only by derepressing hmsT but also by activating hmsD, thereby activating biofilm development.

The Rcs system might respond to different environmental signals, resulting in precise regulation of biofilm formation through control of hmsT and hmsD. In Y. pseudotuberculosis, RcsA and RcsB could respond to different environmental signals respectively and then precisely regulate hmsCDE transcription, which in turn control biofilm formation to adapt different environment. In Y. pestis, HmsT and HmsD are differentially regulated by Rcs, which may be partially responsible for the two DGCs played differential roles on environment-dependent biofilm formation.

Methods

Bacterial strains and plasmids. The strains and plasmids used are shown in Supplementary Table S1. For construction of hmsC::lacZ and hmsD::lacZ reporter, the 353-bp upstream region together with the first 7 codons of the hmsC or the 624-bp upstream region together with the first 7 codons of the hmsD were amplified by PCR using KIM6+ chromosome DNA as template, respectively. The DNA fragments were digested with HindIII and BamHI, and cloned into pGPD926, resulting in plasmid pYC287 and pYC487. For construction of hmsC::lacZ and lcrQ::lacZ reporter, the 353-bp upstream region together with the first 7 codons of the hmsC or the 353-bp upstream region together with the first 7 codons of the lcrQ was amplified, digested, and cloned into pGPD926, resulting in plasmid pYC593 and pYC597. RcsB(D56Q) and RcsB(D56E) were generated by overlapping PCR as described previously24.

The Rcs box mutation strains were made by two-step allelic replacement24, the mutation sequences are listed in Supplementary Table S2. Briefly, a 2-kb PCR product containing the promoter of hmsCDE was amplified from Y. pestis KIM6+ and cloned into pUC19. Using the resulting plasmid as template, the Rcs box was mutated using a mutagenic PCR primer, and the product was substituted into the Y. pestis chromosome by allelic replacement24. Oligonucleotide primers used are shown in Supplementary Table S2. All strains were verified by PCR, DNA sequencing or plasmid complementation, as appropriate.

β-galactosidase assays. β-galactosidase activities were measured as previously described23. Overnight cultures of Y. pestis harboring lacZ reporters were diluted to OD600 0.05 and grown in LB broth at room temperature to OD600 about 1.5. ONPG (o-nitrophenyl-β-D-galactopyranoside) was cleaved by lysates of cells at 37°C and expressed in Miller units25. Results from three independent experiments done in triplicate were analyzed.

Quantitative real time PCR. qRT-PCR was carried out as previously described24. Briefly, cells were grown in LB broth overnight and diluted to OD600 0.05 and grown in LB broth at room temperature to OD600 about 0.8. Total RNA was isolated from collected cells using the RNeasy Mini Kit (Qiagen). Residual DNA was removed by treatment with DNase I (Ambion) and confirmed by PCR. cDNA was synthesized from the RNA and used for quantitative PCR on an ABI Prism 7900 Sequence Detection System (Taqman, Applied Biosystems). The quantity of mRNA was normalized relative to the quantity of the reference gene rcr (y1485)23. The ratio of the nitrotetraacetic acid (NTA) His Bind Purification Kit (Novagen), as recommended by the manufacturer.

Electrophoretic mobility shift assay (EMSA). EMSA was performed as previously described. Briefly, a 112-bp PCR product containing the Rcs box or mutated Rcs box of the hmsCDE operon promoter region was amplified using the primers shown in Supplementary Table S2. Purified recombinant protein was added to DNA binding reaction mixtures containing 50 mmol Tris-HCl pH 7.5, 100 mmol NaCl, 10 mmol DTT, 500 µg/ml bovine serum albumin (BSA) and 100 ng PCR products. The binding assays were performed in a volume of 16 µl at room temperature for 30 min. After incubation samples were electrophoresed at 70 V for 1.5 hour in 6% DNA retardation gels. The gels were stained with ethidium bromide.

In vitro biofilms. Microtiter plate biofilm assays were carried out as previously described24. Briefly, bacteria were cultured overnight in LB broth supplemented with 4 mmol CaCl2 and 4 mmol MgCl2 overnight and diluted to OD600 0.05 and grown to OD600 about 1.5. ONPG was stained with crystal violet, solubilized with 80% ethanol-20% acetone and measured by spectrophotometry. Triplicate were analyzed.

Results from three independent experiments done in triplicate were analyzed by one-way ANOVA with Bonferroni’s test.

Western blotting. Western blotting was carried out as previously described26. Samples were extracted from same amount of stationary phase cells cultured in 26°C, separated on 10% SDS-PAGE gels, transferred to PVDF membrane (Millipore), analyzed by immunoblot with antibodies to the flag (Invitrogen) or Myc (Invitrogen), and detected with Immobilon Western HRP Substrate (Millipore). Results were quantitated by densitometry using NIH Image.

Supplementary Table S2. Results from three independent experiments done in triplicate were analyzed by one-way ANOVA with Bonferroni’s test.

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Author contributions
Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. prepared figures 1–6. Y.S. and X.G. wrote the main manuscript text. X.G. and G.R. prepared figures 1–6. H.Z. and G.R. preparedfigures 1–6.