The *Yersinia pestis* gcvB gene encodes two small regulatory RNA molecules
Sarah D McArthur, Sarah C Pulvermacher and George V Stauffer*

Address: Department of Microbiology, The University of Iowa, Iowa City, Iowa 52242, USA
Email: Sarah D McArthur - sdmcarthur5@yahoo.com; Sarah C Pulvermacher - sarah-pulvermacher@uiowa.edu; George V Stauffer* - george-stauffer@uiowa.edu
* Corresponding author

Abstract

**Background:** In recent years it has become clear that small non-coding RNAs function as regulatory elements in bacterial virulence and bacterial stress responses. We tested for the presence of the small non-coding GcvB RNAs in *Y. pestis* as possible regulators of gene expression in this organism.

**Results:** In this study, we report that the *Yersinia pestis* KIM6 gcvB gene encodes two small RNAs. Transcription of gcvB is activated by the GcvA protein and repressed by the GcvR protein. The gcvB-encoded RNAs are required for repression of the *Y. pestis* dppA gene, encoding the periplasmic-binding protein component of the dipeptide transport system, showing that the GcvB RNAs have regulatory activity. A deletion of the gcvB gene from the *Y. pestis* KIM6 chromosome results in a decrease in the generation time of the organism as well as a change in colony morphology.

**Conclusion:** The results of this study indicate that the *Y. pestis* gcvB gene encodes two small non-coding regulatory RNAs that repress dppA expression. A gcvB deletion is pleiotropic, suggesting that the sRNAs are likely involved in controlling genes in addition to dppA.

Background

*Yersinia pestis* is the causative agent of plague, an infectious disease that results in lymphatic and blood infections [1]. The *Y. pestis* genome has been sequenced [2,3]. *Y. pestis* carries three plasmids of approximately 9.5, 70, and 100 kilobasepairs and each carries genes necessary for or that contribute to the pathogenicity of the bacterium [1]. The 70 kilobasepair plasmid encodes the low-calcium response stimulon (LCRS). Components of the LCRS include Yops (secreted anti-host proteins) and a type III secretion apparatus, or Ysc. The type III secretion apparatus is responsible for the translocation of the Yops to host cells that in turn down-regulate the response of the host phagocytic cells to infection [4]. Natural LCRS-negative mutants of *Y. pestis* occur, resulting in avirulence of the bacteria [1]. Besides the three plasmids, another pathogenicity factor is pigmentation. Cells of *Y. pestis* adsorb hemin at 26°C but not at 37°C and are pigmented (Pgm*) and virulent. Spontaneous nonpigmented (Pgm-) mutants of *Y. pestis* have been isolated. The Yersiniabactin iron transport system is part of the pgm locus, and its loss results in a Pgm- mutant that is avirulent in mice unless hemin, ferrous sulfate, or ferric chloride is injected into mice along with the bacterial challenge [1].
Recently, a new class of molecules has been shown to regulate gene expression in bacteria, small non-coding regulatory RNAs (sRNAs). These sRNAs have gained much attention as recent genome-wide studies have identified sRNAs in a wide variety of organisms [5]. Most of these bacterial sRNAs are between 50 and 400 nucleotides (nts) in length and play important roles in global regulation [6,7]. Hfq is a small RNA binding protein and sRNAs in particular are targets for Hfq [6]. Binding of these sRNAs by Hfq in some way facilitates base pairing between the sRNAs and their respective target RNAs [8,9]. In *Vibrio cholerae*, sRNAs (Qrr RNAs) have been shown to regulate virulence genes [10] and in *Brucella abortus* an *hfq* mutation is lethal [9]. These results suggest that sRNAs and Hfq likely play important roles in the virulence of certain Gram-negative pathogens.

The *E. coli gcvB* gene encodes sRNAs that are not translated in vivo [11]. A strain carrying a deletion of *gcvB* has constitutive synthesis of OppA and DppA, periplasmic binding proteins of the two major peptide transport systems normally repressed in cells grown in rich medium [12-14]. In addition to OppA and DppA, several other proteins were shown to increase or decrease in response to GcvB RNA levels, but the specific proteins were not identified [11]. Nevertheless, the results show that the GcvB RNAs are regulatory and possibly serve as global regulators. A computer search of the *Y. pestis* sequence showed that *Y. pestis* has a *gcvB* gene that shares considerable sequence homology with the *E. coli* *gcvB* sequence (Fig. 1). Thus, the GcvB RNAs from *E. coli* likely have functional counterparts in *Y. pestis*. The results of this study show that the *Y. pestis* *gcvB* gene encodes two sRNAs that, in turn, have regulatory activity. In addition, a deletion of the *gcvB* gene from the *Y. pestis* chromosome alters growth rate and colony morphology.

**Results and discussion**

**Identification of the *Y. pestis* gcvB gene**

The *E. coli* *gcvB* gene is divergently transcribed from *gcvA*, which encodes the activator protein for *gcvB* expression (Fig. 1) [11]. Thus, we used computer searches of genome

---

**Figure 1**

Comparison of the *E. coli* and *Y. pestis* gcvA/gcvB control regions and gcvB genes. Ec, *E. coli*; Yp, *Y. pestis*. Bases that are identical are boxed in gray. The *E. coli* promoter -10 and -35 sequences are underlined for gcvA [29] and overlined for gcvB [11]. Arrows indicate transcription start sites and directions of transcription of gcvA and gcvB. The GcvA binding region is indicated above the sequence [30]. The deduced *Y. pestis* -10 and -35 promoter sequences are underlined for gcvA and overlined for gcvB, and the deduced GcvA binding site is indicated above the sequence. Two Rho-independent transcription terminators for the *E. coli* and *Y. pestis* *gcvB* genes are indicated by inverted arrows. The fusion points for transcriptional fusions *gcvB*<sup>153:</sup>*lacZ* (TF-1), *gcvB*<sup>164:</sup>*lacZ* (TF-2) and *gcvB*<sup>251:</sup>*lacZ* (TF-3) are indicated by vertical arrows.

---
sequences with the gcvA gene product as a query to predict gcvB homologs in other organisms. We identified GcvB-like RNA sequences in the genera *Yersinia*, *Salmonella*, *Hae-
mophilus*, *Vibrio*, *Pasteurella*, *Shigella*, *Erwinia*, *Klebsiella*, *Photorhabdus* and *Actinobacillus*. Despite considerable sequence variation in many of these homologs, they are predicted by the mfold algorithm [15] to assume a similar secondary structure. A comparison of three of these GcvB RNAs is shown in Fig. 2. The location of the putative *Y. pestis* gcvB gene adjacent to and divergent from gcvA, its 77% sequence similarity to the *E. coli* gcvB sequence and its predicted secondary structure make it a likely homolog of gcvB in *Y. pestis*. Furthermore, identical gcvB sequences can be found in all other *Y. pestis* strains presently in the data base, both virulent and avirulent strains.

**The *Y. pestis* gcvB gene encodes two sRNAs**

The *E. coli* gcvB gene encodes two sRNA transcripts that are not translated in vivo [11]. To determine if the gcvB gene in *Y. pestis* is functional and possibly encodes sRNAs, we initially constructed plasmid pgcvBYP+53::lacZ, carrying a transcriptional fusion of the gcvB gene at basepair (bp) +53 to lacZ. Plasmid pgcvBYP+53::lacZ and the vector alone were transformed into *Y. pestis* strain KIM6, the transformants grown in heart-infusion broth (HIB) + ampicillin (AP) to mid-log phase of growth and the cultures assayed for β-galactosidase activity. The KIM6 and KIM6[pMC1403] control transformants gave 6 ± 0.3 and 6 ± 1 units of β-galactosidase activity, respectively, whereas the KIM6[pgcvBYP+53::lacZ] transformant gave 5,985 ± 118 units of β-galactosidase activity. The results suggest that the gcvB gene is expressed in *Y. pestis***.

The gcvB gene from *Y. pestis* possesses two possible Rho-independent transcription terminators, which if functional, would allow the production of two sRNAs of about 130 nts and 206 nts (Fig. 1). Three transcriptional gene fusions of the *Y. pestis* gcvB gene to lacZ were created to determine if these putative terminator sites function as transcription terminators in *vivo*. The three fusions, designated λgcvBYP+53::lacZ, λgcvBYP+164::lacZ and λgcvBYP+251::lacZ, were used to lysogenize *E. coli* strain GS162. The lysogens were then grown in Luria-Bertani broth (LB) [16] to mid-log phase of growth and the cultures assayed for β-galactosidase activity. About 44% of the β-galactosidase activity seen when the fusion precedes both terminators (λgcvBYP+53::lacZ) is lost when the fusion point follows terminator t1 (λgcvBYP+164::lacZ), implicating t1 as a site of transcription termination in *vivo* (Table 1). The remaining activity that escapes termination by t1 is not seen in GS162λgcvBYP+251::lacZ, indicating t2 also functions as a terminator in *vivo* (Table 1). When the 206 nts preceding terminator t2 for gcvB were analyzed, there were only short open reading frames (ORFs) that could encode polypeptides of 36 amino acids or less. These ORFs all lack good translational start sites and were not tested to determine if they encode small polypeptides. The *E. coli* GcvB RNAs are not translated into polypeptides [11]. Thus, we conclude that the products of the gcvB gene in *Y. pestis* are two sRNAs that are not translated, although

![Secondary structures of GcvB RNAs with 77% (*Y. pestis*) and 53% (*V. cholerae*) identity to the *E. coli* GcvB RNA as predicted by the mfold algorithm [15].](image_url)

**Figure 2**

Secondary structures of GcvB RNAs with 77% (*Y. pestis*) and 53% (*V. cholerae*) identity to the *E. coli* GcvB RNA as predicted by the mfold algorithm [15].
the results do not completely rule out the possibility that the Y. pestis GcvB sRNAs encode small peptides. In E. coli, about 90% of the transcripts that initiate at the gcvB promoter terminate at terminator t1, and the remaining 10% terminate at terminator t2 [11]. A comparison of the E. coli and Y. pestis t1 sites shows that an additional 2 bps occur between the predicted GC-rich stem-loop structure and the run of T residues in the Y. pestis t1 site that are not present in the E. coli sequence, suggesting that the Y. pestis t1 site is likely less functional as a transcription terminator than the E. coli t1 site (Fig. 1). Nevertheless, the results are consistent with the Y. pestis gcvB gene encoding two sRNA molecules of about 130 and 206 nts and in roughly equal amounts.

We used Northern blotting to confirm that the gcvB locus in Y. pestis encodes sRNA transcripts of about 130 and 206 nts. Two small RNA molecules were detected in RNA isolated from Y. pestis KIM6 grown in HIB medium using a probe specific for the gcvB locus (Fig. 3). These results are consistent with the in vivo results with the Y. pestis gcvB transcriptional fusions.

**Regulation of the Y. pestis gcvB gene**

The E. coli gcvB gene is activated by GcvA in the presence of glycine and repressed by GcvA + GcvR in its absence; this repression is enhanced by the addition of purines [11]. The regulation of the Y. pestis gcvB gene was tested with respect to the effects of glycine and purine supplementation to the growth medium and with respect to the GcvR and GcvA proteins and the GcvB RNAs. For these experiments we used the λgcvB<sup>53</sup>::lacZ fusion to lysogenize appropriate E. coli host strains. The lysogens were grown in glucose minimal (GM) or GM supplemented with glycine or inosine to mid-log phase of growth and assayed for β-galactosidase levels. In the wild-type (WT) GS162λgcvB<sup>53</sup>::lacZ lysogen, the addition of glycine to GM growth medium resulted in an 11.5-fold induction of β-galactosidase expression, whereas the addition of the purine inosine resulted in a 2.5-fold repression below the unsupplemented GM level (Table 2, line 1). In the gcvA mutant lysogen GS1118λgcvB<sup>53</sup>::lacZ, the β-galactosidase levels were low and non-inducible by glycine (Table 2, line 2). The addition of inosine had no significant effect in the gcvA mutant strain. In the gcvR mutant lysogen GS1053λgcvB<sup>53</sup>::lacZ, the β-galactosidase levels are constitutively high under all three growth conditions (Table 2, line 3). The results suggest that activation of the Y. pestis gcvB gene requires the GcvA protein and that repression requires the GcvR protein. Whether the negative regulation by GcvR requires a direct interaction of GcvR with GcvA as in E. coli [17,18] awaits further investigation. Furthermore, there appears to be no autoregulation of gcvB by its own sRNA products as the gcvB mutant lysogen GS1144ΔgcvB<sup>53</sup>::lacZ shows normal regulation of the gcvB<sup>53</sup>::lacZ fusion (Table 2, line 4).

**Y. pestis gcvA encodes an activator protein for gcvB expression**

Since activation of the Y. pestis gcvB<sup>53</sup> fusion in E. coli was dependent on GcvA (Table 2), we determined if the Y. pestis gcvA gene also encodes an activator protein for gcvB expression. We assumed this would be the case, as the E. coli and Y. pestis GcvA proteins are 88% identical at the

![Figure 3](image-url)  
Northern blot analysis of GcvB from Y. pestis strain KIM6. Total cell RNA was isolated from strain KIM6 grown in HIB at 30°C to an O.D.<sub>600</sub> of 0.7 and probed with a <sup>32</sup>P-labeled GcvB specific DNA probe as described in Methods. Two gcvB transcripts of about 206 and 130 nucleotides identified are indicated with arrows. Their sizes were determined based on their mobilities relative to the mobility of the E. coli GcvB RNA and 5S rRNAs (not shown).
The Y. pestis gcvA gene encodes a repressor protein for gcvB expression

Since deletion of the gcvR gene in E. coli results in constitutive expression of the Y. pestis gcvB::lacZ fusion (Table 2), we tested if the Y. pestis gcvR gene encodes a repressor for gcvB expression. We assumed this would be the case, as the E. coli and Y. pestis GcvR proteins are 75% identical at the amino acid sequence level. The Y. pestis gcvA gene was cloned into plasmid pACYC177 and tested for its ability to complement an E. coli gcvA mutant. The E. coli strain GS1132 carries a deletion of the gcvA gene [11]. This strain was lysogenized with an E. coli ΔgcvB::lacZ transcriptional gene fusion and subsequently transformed with the control plasmid pACYC177, or pACYC177 carrying either the E. coli or the Y. pestis gcvA gene. The cells were grown in LB to mid-log phase of growth and assayed for β-galactosidase activity. Expression of the E. coli gcvB::lacZ fusion was constitutive in the absence of a functional GcvR protein (Table 4, lines 1 and 2). The gcvB::lacZ fusion, however, was repressed in the presence of either pGS601, carrying E. coli gcvR, or pGcvRΔp177, carrying Y. pestis gcvR (Table 4, lines 3 and 4).

In E. coli, the GcvA and GcvR proteins interact to form a repressor complex [17,18]. The above results suggest that the Y. pestis GcvR protein interacts with the E. coli GcvA protein to form a repression complex. We tested if the Y. pestis gcvA and gcvR gene products also likely form a repressor complex to control expression of an E. coli gcvB::lacZ fusion. Strain GS1131ΔgcvB::lacZ carries ΔgcvR ΔgcvA mutations. Strain GS1131ΔgcvB::lacZ was transformed with plasmid pGcvAΔp177, pGcvRΔp322, or both plasmids. The vectors for pGcvAΔp177 and pGcvRΔp322 are pACYC177 and pBR322, respectively, to insure an excess of GcvRΔp versus GcvAΔp. The cells were grown in GM media + appropriate antibiotics, harvested in mid-log phase of growth and assayed for β-galactosidase activity. The Y. pestis gcvA gene complemented the ΔgcvA mutation, resulting in activation of the gcvB::lacZ fusion (Table 4, line 7). The Y. pestis gcvR gene complemented the gcvR mutation, as repression of the gcvB::lacZ fusion occurred in the pGcvAΔp177 pGcvRΔp322 double transformant (Table 4, line 8). These results suggest that the GcvA and GcvR proteins likely interact to form a repression complex in Y. pestis. In E. coli, GcvA also activates the gcvTHP operon and GcvA + GcvR repress the operon [17,18].

### Table 2: Regulation of the Y. pestis gcvB::lacZ transcriptional fusion in E. coli. Cells were grown in GM media with the indicated supplements to an OD600 of ~0.5 and assayed for β-galactosidase activity [16]. Activity is expressed in Miller units.

| Lysogen | Relevant genotype | β-galactosidase activity for cells grown in: | | |
|---------|------------------|---------------------------------------------|--|--|
| GS162ΔgcvB+53::lacZ | WT | 15 ± 2 | 173 ± 2 | 6 ± 3 |
| GS1118ΔgcvB+53::lacZ | ΔgcvA | 2 ± 1 | 3 ± 1 | 2 ± 1 |
| GS1033ΔgcvB+53::lacZ | gcvR | 620 ± 58 | 419 ± 13 | 440 ± 174 |
| GS1144ΔgcvB+53::lacZ | ΔgcvB | 10 ± 2 | 140 ± 24 | 6 ± 2 |

### Table 3: The Y. pestis gcvA gene encodes an activator protein. Cells were grown in LB to an OD₆₀₀ of ~0.5 and assayed for β-galactosidase activity [16]. Activity is expressed in Miller units.

| Lysogen | Relevant genotype | β-Galactosidase activity |
|---------|------------------|--------------------------|
| GS1132ΔgcvB::lacZ | Δ(gcvA gcvB) | <1 |
| GS1132ΔgcvB::lacZ[pACYC177] | ΔgcvB | <1 |
| GS1132ΔgcvB::lacZ[pGS333] | Δ(gcvB::gcvAΔp) | 399 ± 22 |
| GS1132ΔgcvB::lacZ[pGcvAΔp-p177] | Δ(gcvA gcvB::gcvAΔp) | 254 ± 21 |
The Y. pestis gcvB RNAs are regulatory molecules. However, the mechanism of GcvB RNA repression of dppA has not been determined. Although there is a region of 13–14 nucleotides in the Y. pestis GcvB RNA that can potentially base-pair with both the E. coli and Y. pestis dppA mRNAs near their ribosome binding sites, further studies are necessary to determine if base-pairing of GcvB RNA and dppA mRNA is part of the regulatory mechanism. Furthermore, in E. coli, the 206 nucleotide GcvB RNA is required for repression of oppA and dppA [11]. We are constructing a plasmid that will only produce the 130 nucleotide Y. pestis GcvB RNA to determine whether the 130 or 206 nucleotide RNA species is required for activity in Y. pestis.

### Table 4: The Y. pestis gcvR gene complements an E. coli gcvR mutation. Cells were grown in GM media to an OD$_{600}$ of ~0.5 and assayed for $\beta$-galactosidase activity [16]. Activity is expressed in Miller units.

| Lysogen | Relevant genotype | $\beta$-Galactosidase activity |
|---------|------------------|-------------------------------|
| GS1053::gcvB::lacZ | GcvR | 308 ± 19 |
| GS1053::gcvB::lacZ[pACYC177] | GcvR | 384 ± 175 |
| GS1053::gcvB::lacZ[pGS601] | gcvR/gcvR$^C$ | 11 ± 1.5 |
| GS1053::gcvB::lacZ[pGcvR$^R_\Lambda$]$^{p=177}$ | gcvR/gcvR$^R_\Lambda$ | 14 ± 1.2 |
| GS1131::gcvB::lacZ | ΔgcvA ΔgcvR | 2.2 ± 0.2 |
| GS1131::gcvB::lacZ[pGcvR$^R_\Lambda$]$^{p=322}$ | ΔgcvA ΔgcvR/gcvR$^R_\Lambda$ | 2.8 ± 0.1 |
| GS1131::gcvB::lacZ[pGcvA$^R_\Lambda$]$^{p=177}$ | ΔgcvA ΔgcvR/gcvA$^R_\Lambda$ | 393 ± 8 |
| GS1131::gcvB::lacZ[pGcvA$^R_\Lambda$]$^{p=322}$ | ΔgcvA ΔgcvR/gcvA$^R_\Lambda$ gcvR$^R_\Lambda$ | 6.8 ± 0.4 |

Whether the Y. pestis GcvA and GcvR proteins also regulate the Y. pestis gcv/THP operon, or have additional regulatory roles, awaits further investigation.

The Y. pestis GcvB RNAs regulate the E. coli and Y. pestis dppA genes

The E. coli gcvB gene negatively regulates the dppA and oppA genes [11]. In addition, many other genes were shown to be either negatively or positively regulated by the GcvB RNAs [11]. Thus, the E. coli GcvB RNAs are likely global regulators of gene expression. Y. pestis has homologs of dppA and oppA. To determine if the Y. pestis GcvB RNAs are regulatory, we transformed an E. coli Δ dppA lacZ::lacZ lysogen with pGcvB$^{V_Y}$::p322, the transformant and the parent lysogen were grown in LB to mid-log phase of growth and assayed for $\beta$-galactosidase levels. As expected, deletion of gcvB caused an increase in dppA::lacZ expression (Table 5, line 2). However, pGcvB$^{V_Y}$::p322 complemented the E. coli Δ gcvB mutation, repressing the E. coli dppA::lacZ fusion (Table 5, line 3). Thus, the Y. pestis GcvB RNAs regulate the E. coli dppA::lacZ fusion. We then tested the regulatory activity of the GcvB RNAs in Y. pestis directly. A single-copy plasmid carrying a Y. pestis dppA::lacZ fusion was used to transform Y. pestis strain KIM6 and KIM6Δ gcvB. The transformants were grown in HIB + AP to mid-log phase of growth and assayed for $\beta$-galactosidase levels. Deletion of the gcvB gene resulted in a 7.3-fold increase in dppA::lacZ expression (Table 5, compare lines 4 and 5). The results suggest that the Y. pestis GcvB RNAs are regulatory molecules. However, the mechanism of GcvB RNA repression of dppA has not been determined. Although there is a region of 13–14 nucleotides in the Y. pestis GcvB RNA that can potentially base-pair with both the E. coli and Y. pestis dppA mRNAs near their ribosome binding sites, further studies are necessary to determine if base-pairing of GcvB RNA and dppA mRNA is part of the regulatory mechanism. Furthermore, in E. coli, the 206 nucleotide GcvB RNA is required for repression of oppA and dppA [11]. We are constructing a plasmid that will only produce the 130 nucleotide Y. pestis GcvB RNA to determine whether the 130 or 206 nucleotide RNA species is required for activity in Y. pestis.

### Table 5: Regulation of E. coli and Y. pestis dppA::lacZ translational gene fusions by the Y. pestis gcvB gene. Cells were grown in LB (E. coli) or in HIB (Y. pestis) at 37°C to an OD$_{600}$ of ~0.5 and assayed for $\beta$-galactosidase activity [16]. Activity is expressed in Miller units. The parent strains KIM6 and KIM6Δ gcvB grown in HIB at 37°C showed <5 units of $\beta$-galactosidase activity.

| Lysogen | Relevant genotype | $\beta$-Galactosidase activity |
|---------|------------------|-------------------------------|
| GS162::dppA$^{E_C}$::lacZ | WT | 103 ± 24 |
| GS1144::dppA::lacZ | ΔgcvB | 554 ± 81 |
| GS1144::dppA$^{E_C}$::lacZ[pGcvB$^{V_Y}$::p322] | ΔgcvB/gcvB$^{V_Y}$ | 154 ± 32 |
| KIM6[pdppA$^{p=177}$::lacZ] | WT | 62 ± 13 |
| KIM6Δ gcvB[pdppA$^{p=177}$::lacZ] | ΔgcvB | 455 ± 7 |
have no observable phenotype. The KIM6Δ gcvB strain also showed a different colony morphology from WT KIM6. WT KIM6 colonies appear smooth and sticky, whereas the KIM6Δ gcvB colonies appear dry and compact. The presence of pgcvBYP-57 in KIM6ΔgcvB again complemented the gcvB deletion, as the phenotype was restored back to the WT colony morphology.

In E. coli, many genes respond to the GcvB RNAs [11]. The pleiotropic nature of the Y. pestis gcvB deletion suggests that the Y. pestis GcvB RNAs are likely global regulators as well. Identification of the specific genes regulated by the GcvB RNAs will allow us to test directly their involvement in virulence. GcvB RNAs that are responsible for the altered phenotype were carried at 300 μg ml⁻¹ and 50 μg ml⁻¹, respectively. For Y. pestis strains, HIB was used [21]. Agar was added at 1.5% to make solid media. Antibiotics were added at the following concentrations: AP, 150 μg ml⁻¹ for multi-copy plasmids and 50 μg ml⁻¹ for single-copy plasmids; chloramphenicol (CM), 20 μg ml⁻¹; tetracycline (TC), 10 μg ml⁻¹.

β-galactosidase assays
β-galactosidase assays were performed on mid-log phase cells (OD₆₀₀~0.5) as described by Miller [16]. Each experiment was repeated at least twice, with each sample assayed in triplicate.

DNA manipulation
Plasmid DNA was isolated using Qiagen Miniprep kits as described by the manufacturer (Qiagen). Restriction enzyme digestions and DNA ligations were carried out according to the manufacturer (New England Biolabs). DNA sequencing was performed by the University of Iowa DNA Core Facility.

PCR
PCR reactions were performed in 100 μl volumes. Each reaction mixture contained 10 μl 10 × polymerase buffer, 10 μl 10 × dNTPs (0.2 mM each), 5 μl Y. pestis DNA (~15 ng), 100 pmoles of forward and reverse primers designed specifically for each reaction, 1 μl of vent polymerase, and sterile water to bring the volume to 100 μl. PCR reactions were carried out under the following conditions: 5 min pre-incubation at 95°C, and then 30 cycles of 95°C for 30 sec, 45°C for 30 sec, and 72°C for 2 min.

RNA extraction and Northern blot analysis
Y. pestis KIM6 was grown in HIB at 30°C to an O.D₆₀₀ of 0.7, the cells collected for 1 minute in a microcentrifuge and immediately frozen at -70°C. Total cellular RNA was isolated using the MasterPure™ RNA purification kit (Epicenter). The final RNA pellet was re-suspended in water treated with diethyl pyrocarbonate and kept at -70°C. The RNA concentration was measured with a spectrophotometer at 260 nm. RNA (10 μg) was separated through a 1.5% formaldehyde gel and blotted on to a Biodyne Plus
Table 6: Bacterial strains, plasmids and phage. All *E. coli* strains listed also carry Δ(argF-lac)U169, pheA905, thi, araD129, rpsL150, relA1, deoC1, fib301, ptsF25 and rpsR mutations.

| Strains/plasmids/phage | Relevant genotype | Source/reference |
|------------------------|-------------------|-----------------|
| GS162                  | WT                | This laboratory  |
| GS1032                 | ΔgcvR::Tn10        | [19]            |
| GS1118                 | ΔgcvA::aadA        | This laboratory  |
| GS1131                 | ΔgcvA::aadA ΔgcvR::ΣKΔR | [11] |
| GS1132                 | ΔgcvA gcvB::aadA   | [11]            |
| GS1144                 | ΔgcvB::ΣCMRΔR     | This laboratory  |
| KIM6                   | lcr-              | [31]            |
| KIM6ΔgcvB              | ΔgcvB::ΣCMRΔR     | This study       |
| Plasmid                |                   |                 |
| pGS336                 | Single-copy trans| This laboratory  |
| pgsBVp-p322            | tional lacZ fusion vector |          |
| pgsBVp-ac              | Carries *Y. pestis* gcvB in a single-copy vector | This study |
| pgsBVp-p177            | Carries *Y. pestis* gcvA in pACYC177 | This study |
| pgsBVp-p177            | Carries *Y. pestis* gcvR in pACYC177 | This study |
| pgsBVp-p322            | Carries *Y. pestis* gcvR in pBR322 | This study |
| pdsrvA::lacZ           | *Y. pestis* dppA::lacZ fusion in pGS336 | This study |
| pGS335                 | Carries *E. coli* gcvA in pACYC177 | This lab |
| pGS601                 | Carries *E. coli* gcvR in pACYC177 | This lab |
| Phage                  |                   |                 |
| λdppA::lacZ            | λgt2 with *E. coli* dppA::lacZ translational fusion | [11] |
| λgcvB::lacZ            | λgt2 with *E. coli* gcvB::lacZ transcriptional fusion | [11] |
| λgcvB+53::lacZ         | λgt2 with *Y. pestis* gcvB+53::lacZ transcriptional fusion | This study |
| λgcvB+164::lacZ        | λgt2 with *Y. pestis* gcvB+164::lacZ transcriptional fusion | This study |
| λgcvB+251::lacZ        | λgt2 with *Y. pestis* gcvB+251::lacZ transcriptional fusion | This study |

Membrane (ISC BioExpress). The blot was hybridized with a PCR generated DNA fragment from bp +1 to +198 of the *Y. pestis* gcvB gene and 32P-labeled using the Rediprime® II Random Prime Labeling System (Amersham Biosciences). Hybridization of the blot was at 58°C as described [22].

**Construction of gcvA, gcvB and gcvR plasmids**

The *Y. pestis* gcvB gene was cloned as follows. PCR primer YP-GCVB1F has an artificial EcoRI site and is complementary to the *Y. pestis* KIM6 DNA sequence beginning 114 bases upstream of the gcvB transcription start site. PCR primer YP-GCVB2R has an artificial HindIII site and is complementary to the *Y. pestis* DNA sequence beginning 45 bases downstream of the gcvB transcriptional termination site t2 (Fig. 1). Following PCR amplification, using *Y. pestis* chromosomal DNA as template, the amplified DNA was digested with EcoRI and HindIII, the 400 bp fragment carrying gcvB isolated from a 1% agarose gel and ligated into the EcoRI and HindIII sites of plasmid pBR322 [23], generating plasmid pgsBVp-p322. The *Y. pestis* gcvA and gcvR genes were cloned using a similar strategy. For gcvA, both the forward and reverse primers contained artificial HindIII sites complementary to the *Y. pestis* sequence beginning 111 bases upstream of the gcvA transcription start site and 349 bases downstream of the gcvA translation stop codon. For gcvR, both the forward and reverse primers contained artificial HindIII sites complementary to the *Y. pestis* sequence beginning 313 bases upstream of the gcvR transcription start site and 198 bases downstream of the gcvR translation stop codon. The PCR amplified fragments were cloned into the HindIII site of plasmid pACYC177 [24], generating plasmids pgsAVp-p177 and pgsRvir-p177. In a second construct of gcvR, both the upstream and downstream primers contained artificial EcoRI sites and the PCR amplified fragment was cloned into the EcoRI site of plasmid pBR322, generating plasmid pgsRvir-p322. Each gene was sequenced at the University of Iowa DNA Core Facility to verify that no bp changes were introduced during the PCR amplification procedure.

**Construction of lacZ gene fusions**

Three different transcriptional gene fusions of gcvB to the lacZ gene were constructed by PCR synthesis of fragments with common BamHI termini 128 bp upstream of the gcvB transcription start site and 3 different fusion points within gcvB. In plasmid pBtp-p53::lacZ, the downstream PCR primer hybridized to the gcvB sequence beginning at
bp +53 relative to the predicted transcription start site (+1) of gcvB (Fig. 1). A synthetic HindIII site was included at the end of the primer to allow the cloning of the 202 bp BamHI-HindIII fragment into the BamHI-HindIII sites of the lacZ transcriptional reporter plasmid pQF50 [25]. Plasmids pBpB +164::lacZ and pBpB +251::lacZ were constructed similarly except that the downstream primers used for PCR synthesis hybridized to the gcvB sequences beginning at bp +164 and +251 (Fig. 1), and the 313 and 400 bp fragments produced were cloned into the BamHI-HindIII sites of pQF50. Each fusion was sequenced at the University of Iowa DNA Core Facility to verify that the fusions were at the correct sites and that no bp changes were introduced during the PCR amplification procedure. Each gcvB transcriptional fusion was then subcloned into plasmid pMC1403 [26], generating plasmids pgcvBp +53::lacZ, pgcvBp +164::lacZ, and pgcvBp +251::lacZ, and subsequently transferred to phase λgt2 [27] as described [11], generating phage λgcvBp +53::lacZ, λgcvBp +164::lacZ and λgcvBp +251::lacZ, respectively.

A single-copy Y. pestis dppA::lacZ translational fusion was constructed in two steps. First, a dppA::lacZ translational fusion was constructed using an upstream PCR primer with an EcoRI site complementary to the Y. pestis DNA sequence beginning 300 bps upstream of the dppA transcription initiation site and a downstream primer that contains an artificial SmaI site and that hybridizes to the dppA sequence after the 15th codon relative to the translation initiation site. The 611 bp dppA fragment was cloned into the EcoRI and SmaI sites of the lacZ translational reporter plasmid pMC1403. The fusion was sequenced at the University of Iowa DNA Core Facility to verify that the fusion was at the correct site and that no bp changes were introduced during the PCR amplification procedure. The dppA::lacZ fusion, along with the lacY and lacA genes, was then cloned into the single-copy plasmid pGS366, designated pdpdA::lacZ.

**Chromosomal deletion of gcvB**

A gcvB deletion was constructed on the Y. pestis chromosome essentially as described [28]. Y. pestis strain KIM6 was transformed with plasmid pKD46, which encodes the Red recombinase of phage λ [28]. PCR products were then generated using two primers with 50 nt extensions that are complementary to sequences that flank the gcvB gene and 20 nt priming sequences that are complementary to the template plasmid pKD32 and that flank the CMR gene and the FLP recognition sequence [28]. The PCR fragment was gel purified and used to transform Y. pestis KIM6 [pKD46]. The cells were plated on HIB plates with CM and CMR recombinants were selected. One CMR recombinant was single colony purified, chromosomal DNA was prepared, and PCR analysis was used to verify that the gcvB gene was deleted and replaced with the CMR marker. The pKD46 plasmid is a temperature sensitive replicon and was cured by growth at 37°C [28]. The strain was designated KIM6ΔgcvB.

**Authors’ contributions**

SM carried out most of the genetic experiments and wrote the first draft of the manuscript. SP carried out the genetic experiments with gcvR and also performed the Northern analysis. GS carried out the computer search to identify putative gcvB genes in other organisms and was the principal investigator and supervised the project.

**Acknowledgements**

We are indebted to S. Straley for providing Y. pestis strain KIM6. This work was supported by grant GM069506 from the National Institutes of Health.

**References**

1. Perry RD, Fetherston JD: *Yersinia pestis*-etiologic agent of plague. *Clin Microbiol Rev* 1997, 10(1):35-66.
2. Deng W, Burland V, Plunkett G, Boutin A, Mayhew GF, Liss P, Perna NT, Rose DJ, Mau B, Zhou S, Schwartz DC, Fetherston JD, Lindler LE, Brinkac LR, Plano GV, Straley SC, McDonough KA, Nilles ML, Matson JS, Blatner FR, Perry RD: Genome sequence of *Yersinia pestis* KIM. *J Bacteriol* 2002, 184(16):4601-4611.
3. Parkhill J, Wren BW, Thomson NR, Tabil RW, Holden MT, Prentice MB, Sebastian M, James KD, Churcier C, Mungall KL, Baker S, Basham D, Bentley SD, Brooks K, Cerdeno-Tarraga AM, Chillingworth T, Cronin A, Davies RM, Davis P, Dougan G, Feltham T, Hamlin N, Holroyd S, Jenkins K, Karleryavek AV, Leather S, Moule S, Oyston PC, Quail M, Rutherford K, Simmonds M, Skelton J, Stevens K, Whitehead S, Barrett BG. Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature 2001, 413(6853):223-227.
4. Fields KA, Nilles ML, Cowan C, Straley SC. Virulence role of V antigen of *Yersinia pestis* at the bacterial surface. *Infect Immun* 1999, 67(10):5395-5408.
5. Hershberg R, Altvia S, Margulies H: A survey of small RNA-encoding genes in *Escherichia coli*. *Nucleic Acids Res* 2003, 31(7):1813-1820.
6. Wassarman KM, Repolla F, Rosnow S, Storz G, Gottesman S: Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev* 2001, 15(13):1637-1651.
7. Gottesman S: Micros for microbes: non-coding regulatory RNAs in bacteria. *Trends Genet* 2005, 21(7):399-404.
8. Zhang A, Wassarman KM, Ortega J, Steven AC, Storz G: The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol Cell* 2002, 9(1):11-22.
9. Christiansen JK, Larsen MH, Ingmer H, Sogaard-Andersen L, Kallipolitis BT: The RNA-binding protein Hfq of *Listeria monocytogenes*: role in stress tolerance and virulence. *J Bacteriol* 2004, 186(11):3355-3362.
10. Lenz DH, Mok KC, Lelby BN, Kulkarni RV, Wingreen NS, Bassler BL: The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* 2004, 118(1):69-82.
11. Urbanowicz ML, Stauffer LT, Stauffer GV: The *gcvB* gene encodes a small untranslated RNA involved in expression of the dipetide and oligopeptide transport systems in *Escherichia coli*. *Mol Microbiol* 2000, 37(4):856-868.
12. Olson ER, Dunyak DS, Jurss LM, Poorman RA: Identification and characterization of dppA, an *Escherichia coli* gene encoding a periplasmic dipetide transport protein. *J Bacteriol* 1991, 173(1):234-244.
13. Guyer CA, Morgan DG, Stasov JV: Binding specificity of the periplasmic oligopeptide-binding protein from *Escherichia coli*. *J Bacteriol* 1986, 168(2):775-779.
14. Manson MD, Blank V, Bradge G, Higgins CF: Peptide chemotaxis in *E. coli* involves the Tap signal transducer and the dipetide permease. *Nature* 1986, 321(6067):253-256.
15. Mfold algorithm [www.bioinfo.rpi.edu/applications/mfold/old/rna] [http://www.bioinfo.rpi.edu/applications/mfold/old/rna]
16. Miller JH: A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; 1992.
17. Ghrist AC, Heil G, Stauffer GV: GcvR interacts with GcvA to inhibit activation of the Escherichia coli glycine cleavage operon. Microbiology 2001, 147(Pt 8):2215-2221.
18. Heil G, Stauffer LT, Stauffer GV: Glycine binds the transcriptional accessory protein GcvR to disrupt a GcvA/GcvR interaction and allow GcvA-mediated activation of the Escherichia coli gcvTHP operon. Microbiology 2002, 148(Pt 7):2203-2214.
19. Ghrist AC, Stauffer GV: Characterization of the Escherichia coli gcvR gene encoding a negative regulator of gcv expression. J Bacteriol 1995, 177(17):4980-4984.
20. Vogel Hj, Bonner DM: Acetylornithinase of Escherichia coli: partial purification and some properties. J Biol Chem 1956, 218(1):97-106.
21. Kubota K, Yamamoto A: [Tetanus toxin production. I. Peptone-free medium for the toxin production with special reference to the significance of the bovine heart infusion]. Nippon Sakingaku Zasshi 1966, 21(11):651-660.
22. Song Yj, Sinski MF: Effect of the human cytomegalovirus IE86 protein on expression of E2F-responsive genes: a DNA microarray analysis. Proc Natl Acad Sci U S A 2002, 99(9):2836-2841.
23. Bolivar F, Rodriguez RL, Greene Pj, Betlach MC, Heyneker HL, Boyer HW: Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 1977, 2(2):93-113.
24. Chang AC, Cohen SN: Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol 1978, 134(3):1141-1156.
25. Farinha MA, Kropinski AM: Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. J Bacteriol 1990, 172(6):3496-3499.
26. Casadaban MJ, Chou J, Cohen SN: In vitro gene fusions that join an enzymatically active beta-galactosidase segment to amino-terminal fragments of exogenous proteins: Escherichia coli plasmid vectors for the detection and cloning of translational initiation signals. J Bacteriol 1980, 143(2):971-980.
27. Panasenko SM, Cameron JR, Davis RW, Lehman IR: Five hundred-fold overproduction of DNA ligase after induction of a hybrid lambda lysogen constructed in vitro. Science 1977, 196(4286):188-189.
28. Datsenko KA, Wanner BL: One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 2000, 97(12):6640-6645.
29. Wilson RL, Stauffer GV: DNA sequence and characterization of GcvA, a LysR family regulatory protein for the Escherichia coli glycine cleavage enzyme system. J Bacteriol 1994, 176(10):2862-2868.
30. Wilson RL, Urbanowski ML, Stauffer GV: DNA binding sites of the LysR-type regulator GcvA in the gcv and gcvA control regions of Escherichia coli. J Bacteriol 1995, 177(17):4940-4946.
31. Sikkema DJ, Brubaker RR: Outer membrane peptides of Yersinia pestis mediating siderophore-independent assimilation of iron. Biol Met 1989, 2(3):174-184.