Role of the *Escherichia coli* Hfq protein in GcvB regulation of oppA and dppA mRNAs

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The gcvB gene encodes a small non-translated RNA (referred to as GcvB) that regulates oppA and dppA, two genes that encode periplasmic binding proteins for the oligopeptide and dipeptide transport systems. Hfq, an RNA chaperone protein, binds many small RNAs and is required for the small RNAs to regulate expression of their respective target genes. We showed that repression by GcvB of dppA::lacZ and oppA::phoA translational fusions is dependent upon Hfq. Double mutations in gcvB and hfq yielded similar expression levels of dppA::lacZ and oppA::phoA compared with gcvB or hfq single mutations, suggesting that GcvB and Hfq repress by the same mechanism. The effect of Hfq is not through regulation of transcription of gcvB. Hfq is known to increase the stability of some small RNAs and to facilitate the interactions between small RNAs and specific mRNAs. In the absence of Hfq, there is a marked decrease in the half-life of GcvB in cells grown in both Luria–Bertani broth and glucose minimal medium with glycine, suggesting that part of the role of Hfq is to stabilize GcvB. Overproduction of GcvB in wild-type *Escherichia coli* results in superrepression of a dppA::lacZ fusion, but overproduction of GcvB in an hfq mutant does not result in significant repression of the dppA::lacZ fusion. These results suggest that Hfq also is likely required for GcvB–mRNA pairing.

INTRODUCTION

The *Escherichia coli* gcvTHP operon encodes the glycine cleavage enzyme complex that provides one-carbon units for cellular methylation reactions (Kikuchi, 1973). The gcvB gene encodes a small, non-translated RNA (sRNA) of 206 nt (referred to as GcvB) that, in turn, regulates several genes encoding proteins involved in various metabolic pathways. These proteins include OppA and DppA, the periplasmic binding proteins for the oligopeptide and dipeptide transport systems, respectively, as well as SstT, the primary serine transport protein (Urbanowski et al., 2000) (S.C. Pulvermacher and others, unpublished results). The GcvA and GcvR proteins regulate both the gcvTHP operon and the gcvB gene (Stauffer & Stauffer, 1994; Urbanowski et al., 2000). Both gcvTHP and gcvB are activated by GcvA in response to glycine and repressed by GcvA and GcvR together in the absence of glycine; this repression is enhanced in the presence of purines (Stauffer & Stauffer, 1994; Urbanowski et al., 2000; Wilson et al., 1993). Because expression of gcvB follows a pattern of regulation qualitatively similar to the gcvTHP operon, we hypothesize that GcvB plays a role in an integrated cellular response to regulate other genes in conjunction with the glycine cleavage enzyme system.

The *E. coli* chromosome encodes >80 sRNAs that function as regulators in different cellular processes (Argaman et al., 2001; Gottesman, 2004; Hershberg et al., 2003; Wagner & Flardh, 2002; Wassarman et al., 1999, 2001; Wassarman, 2002). Many of the sRNAs regulate expression of target genes post-transcriptionally by complementary base pairing with the target mRNAs in trans. A common characteristic of these sRNAs is the requirement of the RNA chaperone protein, Hfq (Zhang et al., 2003). Hfq is a small, abundant RNA binding protein first described in *E. coli* as host factor I (HF-I), and was found to be necessary for replication of the RNA phage Qβ (Franze de Fernandez et al., 1968); further analyses have shown that Hfq homologues are found in both Gram-positive and Gram-negative bacteria (Moller et al., 1998, 2002). Hfq is a pleiotropic regulator that controls the expression of proteins by affecting the translation, stability or polyadenylation of mRNAs (Gottesman, 2004). Hfq preferentially binds AU-rich sequences in single-stranded regions, usually close to structured regions of RNA (Lease & Belfort, 2000; Moller et al., 2002; Schumacher et al., 2002; Zhang et al., 1998, 2002), and sRNAs in particular are targets for Hfq binding (Wassarman et al., 2001). In *E. coli* Hfq is known to bind the sRNAs OxyS, DsrA, RprA, RyhB, MicC, GadY and Spot42 (Chen et al., 2004; Masse & Gottesman, 2002; Moller et al., 2002; Opdyke et al., 2004; Sledjeski et al., 2001; Vecerek et al., 2003; Wassarman et al., 2001; Zhang et al., 1998). Binding of Hfq to sRNAs is...
predicted to change the secondary structure of some sRNAs, thereby affecting the processing or intracellular stability of the sRNA transcript (Moller \textit{et al}., 2002; Zhang \textit{et al}., 2002). Additionally, binding of Hfq to the sRNA, target mRNA, or both the sRNA and target mRNA may function to facilitate RNA–RNA interactions (Moller \textit{et al}., 2002; Zhang \textit{et al}., 2002). Therefore, it has been proposed that enhancement of RNA–RNA pairing is a general function of Hfq (Vecerek \textit{et al}., 2003; Zhang \textit{et al}., 2002).

Previously we have shown that GcvB regulates both DppA and OppA in LB (Pulvermacher \textit{et al}., 2008; Urbanowski \textit{et al}., 2000), and microarray analysis has indicated that \textit{oppA}, \textit{dppA} and \textit{sstT} mRNAs are negatively regulated by Hfq (Guisbert \textit{et al}., 2007). Hfq has been reported to bind GcvB (Zhang \textit{et al}., 2003), and Hfq has been shown to have a stabilizing effect on GcvB in Salmonella (Sharma \textit{et al}., 2007). Thus, we tested whether Hfq is required for GcvB to regulate \textit{dppA}, \textit{lacZ} and \textit{oppA}:\textit{phoA} in E. coli, as well as the effect of Hfq on GcvB stability. Our results demonstrate that repression of \textit{dppA}, \textit{lacZ} and \textit{oppA}:\textit{phoA} translational fusions is dependent on Hfq. Furthermore, our results suggest that Hfq functions to stabilize GcvB. These results suggest that an Hfq–GcvB interaction is necessary for GcvB-mediated regulation of these genes as well as to facilitate RNA–RNA pairing of GcvB with its target mRNAs.

\section*{METHODS}

\subsection*{Bacterial strains, plasmids and phages.} The \textit{E. coli} strains and plasmids used in this study are listed in Table 1. Strains GS1148 and GS1149 were constructed by transduction. P1\textit{clr} phage grown on the \textit{hfq-1::\textit{Ω}CM\textsuperscript{β}} strain GS081 was used to transduce strains GS162 and GS1132 to chloramphenicol-resistant (CM\textsuperscript{R}), generating strains GS1148 and GS1149, respectively. The \textit{iddppA::lacZ} and \textit{ioppA::phoA} translational fusion phages have been described previously (Pulvermacher \textit{et al}., 2008; Urbanowski \textit{et al}., 2000). The \textit{igcvB::lacZ} transcriptional fusion phage includes 150 bp upstream of the \textit{gcvB} transcription initiation site to bp +50 within \textit{gcvB} fused to translationally competent \textit{lacZYA} genes in phase \textit{λgt2} (Urbanowski \textit{et al}., 2000). These phages were used to lysogenize various \textit{E. coli} host strains as described previously (Urbanowski \& Stauffer, 1986). Each lysogen was tested to ensure that it carried a single copy of the \textit{λ} chromosome by infection with \textit{λd90c17} (Shimada \textit{et al}., 1972). All lysogens were grown at 30 °C since all fusion phages carry the \textit{λ}AB57 mutation, resulting in a temperature-sensitive \textit{λ}I repressor (Panasenko \textit{et al}., 1977).

Plasmid pGS609, carrying the \textit{E. coli} \textit{hfq} gene, was constructed as follows. In a PCR, HindIII primer 1 (5'-CCCGAAGCTTCCGCGCGTGCTACC) hybridized to a region beginning 992 bp upstream of the Hfq translation start site and HindIII primer 2 (5'-CCGCAAGCTTTGATTGGGGCCGCCCGTCTCCGGT) hybridized to a region beginning 109 bp downstream of the Hfq translation stop codon. PCR amplification was carried out using Vent DNA polymerase (New England Biolabs). Cycling conditions were as follows: 1 min 30 s denaturation at 95 °C, 1 min annealing at 55 °C, 2 min extension at 72 °C for 30 cycles. The 1409 bp PCR-generated HindIII fragment was cloned into the HindIII site in plasmid pACYC177 (Chang \& Cohen, 1978), and this was verified by DNA sequence analysis at the DNA Core Facility of the University of Iowa.

\subsection*{Media.} The complex medium used was Luria–Bertani broth (LB) (Miller, 1972). The minimal medium used was the salts of Vogel \& Bonner (1956) supplemented with 0.4\% (w/v) glucose (GM). Agar was added at 1.5\% (w/v) to make solid media. Supplements were added at the following concentrations (μg ml\textsuperscript{-1}): ampicillin (AP), 50; chloramphenicol (CM), 20; glycine, 300; inosine, 50%; phenylalanine, 50; rifampicin, 250; thiamine, 1. GM media were always supplemented with phenylalanine and thiamine, since all strains carry the \textit{pheA905} and \textit{thi} mutations.

\subsection*{Enzyme assays.} β-Galactosidase assays were performed on mid-exponential-phase cells (OD\textsubscript{600}~0.5) as described by Miller (1972) using the chloroform/SDS lysis procedure. Alkaline phosphatase assays were performed on mid-exponential-phase cells (OD\textsubscript{600}~0.5) as described by Brickman \& Beckwith (1975). Results are the averages of two or more assays with each sample done in triplicate.

\subsection*{RNA extraction and Northern blot analysis.} For Northern blots, \textit{E. coli} strains GS162 [wild-type (WT)], GS1148 (\textit{Δhfq}), and GS1148 \textit{[Δhfq (phfq +++)]} (Table 1) were grown in 40 ml LB or GM + glycine (AP was added to the transformant) to the mid-exponential phase of growth. Rifampicin (250 μg ml\textsuperscript{-1}) was added to

\begin{table}[h]
\centering
\caption{Strains and plasmids.}
\begin{tabular}{|c|c|c|}
\hline
Strain or plasmid & Relevant genotype & Source or reference \\
\hline
\textbf{Strains*} & & \\
GS081 & \textit{MCA100 hfq-1::\textit{Ω}CM}\textsuperscript{β} & G. Storz \\
GS162 & WT & This laboratory \ 
GS1132 & \textit{ΔgcvB}\textit{gcvB}::\textit{ΩaadA} (referred to as \textit{ΔgcvAB}) & Urbanowski \textit{et al}., (2000) \ 
GS1144 & \textit{ΔgcvB}::\textit{ΩCM}\textsuperscript{β} (referred to as \textit{ΔgcvB}) & Pulvermacher \textit{et al}., (2008) \ 
GS1148 & \textit{hfq-1::ΩCM}\textsuperscript{β} (referred to as \textit{Δhfq}) & This study \ 
GS1149 & \textit{ΔgcvB}\textit{gcvB}::\textit{ΩaadA hfq-1::ΩCM}\textsuperscript{β} (referred to as \textit{ΔgcvAB Δhfq}) & This study \ 
\textbf{Plasmids} & & \\
pGS341 & Single-copy vector + \textit{gcvA} (\textit{gcvA}\textsuperscript{+}) & Jourdan \& Stauffer (1998) \ 
pGS571 & Multicopy vector + WT \textit{gcvB} (\textit{gcvB}\textsuperscript{+}) & This laboratory \ 
pGS594 & Single-copy vector + WT \textit{gcvB} (\textit{gcvB}\textsuperscript{+}) & This laboratory \ 
pGS609 & Multicopy vector + \textit{hfq} (\textit{hfq}\textsuperscript{+}) & This study \ 
\hline
\end{tabular}
\*All strains, except GS081, also carry the \textit{pheA905 thi araD129 rpsL150 relA1 deoC1 flbB5301 ptsF25} and \textit{rbsR} mutations.
\end{table}
GcvB and Hfq regulation of oppA and dppA mRNAs

RESULTS AND DISCUSSION

Hfq is required for GcvB repression of dppA::lacZ and oppA::phoA

Hfq is an RNA binding protein with structural similarities to eukaryotic Sm proteins (Moller et al., 2002; Zhang et al., 1998), and thus far there is a correlation between sRNAs that act by means of RNA–RNA base pairing and the requirement for Hfq (Masse & Gottesman, 2002). Many sRNAs that regulate by RNA–RNA base pairing require Hfq (Sledjeski et al., 2001; Zhang et al., 1998), and thus far there is a correlation between sRNAs that act by means of RNA–RNA base pairing and the requirement for Hfq (Masse & Gottesman, 2002). To confirm a role for Hfq in regulation by GcvB, we tested whether Hfq affects dppA::lacZ and oppA::phoA expression. WT, ΔgcvB, Δhfq and ΔgcvBΔhfq strains lysogenized with either λdppA::lacZ or λoppA::phoA were grown in LB to mid-exponential phase and assayed for either β-galactosidase or alkaline phosphatase activity. The ΔgcvB strain was used instead of the ΔgcvB::gcvA strain because the ΔgcvB strain carries a spectinomycin-resistance cassette, while ΔgcvB carries a CM-resistance cassette. The spectinomycin cassette in ΔgcvB allowed for the transduction of the hfq−1::CM R allele into the ΔgcvB strain to generate the ΔgcvB Δhfq double mutant. Both ΔgcvB λdppA::lacZ and ΔgcvB λoppA::lacZ produced similar β-galactosidase activity levels and ΔgcvB λoppA::phoA and ΔgcvB λoppA::phoA produced similar alkaline phosphatase activity, suggesting that both strains are similarly regulated (data not shown). Expression of dppA::lacZ in the WT lysogen was 10-fold lower than expression in the ΔgcvB lysogen (Table 2, lines 1 and 2). We observed a similar fold difference in ΔdppA::lacZ β-galactosidase activity between the WT and the ΔgcvB lysogens (data not shown). The deletion of hfq in the Δhfq ΔdppA::lacZ lysogen resulted in constitutive β-galactosidase expression (Table 2, line 3). Expression of dppA::lacZ in the ΔgcvB Δhfq double mutant was slightly elevated compared with either single mutant (Table 2, compare lines 2 and 3 with line 4). Although the levels in the double mutant are not additive, suggesting that GcvB and Hfq are part of the same regulatory mechanism, the higher levels in the double mutant are consistent (>10 independent assays). It is possible that an additional regulatory factor is involved in Hfq-mediated repression of dppA::lacZ or that Hfq regulation of dppA::lacZ is not entirely GcvB-dependent. Expression of oppA::phoA in the WT lysogen was fivefold lower than in the ΔgcvB lysogen (Table 2, lines 8 and 9). Again, the loss of hfq in the Δhfq λoppA::phoA lysogen resulted in constitutive alkaline phosphatase expression (Table 2, line 10). Expression of oppA::phoA in the ΔgcvB Δhfq double mutant was not significantly different from that in either single mutant (Table 2, compare lines 9 and 10 with line 11). Additionally, in Salmonella, elevated OppA protein levels have been observed in both ΔgcvB and Δhfq strains with no additional pronounced increase in OppA protein levels in a ΔgcvB Δhfq double deletion (Sharma et al., 2007). The results suggest that GcvB and Hfq are part of the same regulatory mechanism for oppA::phoA regulation. As a control, the ΔgcvB Δhfq double mutant lysogenized with either λdppA::lacZ or λoppA::phoA was transformed with a single-copy plasmid carrying gcvA and assayed for either β-galactosidase or alkaline phosphatase activity. Because both gcvA and gcvB...
are deleted in strain GS1149, we wanted to be certain that the loss of GcvA is not responsible for the observed altered regulation. Expression of dppA::lacZ and oppA::phoA in the ΔgcvAB Δhfq double mutant transformed with plasmid pgcVA\(^+\) did not show an appreciable difference in β-galactosidase or alkaline phosphatase activity compared with either untransformed lysogen (Table 2, compare lines 4 and 5 and lines 11 and 12). These results are consistent with Hfq and GcvB functioning in the same regulatory mechanism to regulate both dppA::lacZ and oppA::phoA expression.

### Hfq does not regulate gcvB transcription

It is possible that the hfq gene is required for gcvB expression. Thus, the failure to observe repression of dppA::lacZ and oppA::phoA in the Δhfq strain may be due to decreased GcvB levels. Therefore, the WT strain was lysogenized with the ΔgcvB::lacZ phage and transduced to CM\(^+\) with P1 phage grown on GS081 and was designated GS1148 ΔgcvB::lacZ. The WT ΔgcvB::lacZ and Δhfq ΔgcvB::lacZ lysogens were grown in LB to mid-exponential phase and assayed for β-galactosidase activity. The deletion of hfq had no significant effect on gcvB::lacZ transcription (Table 2, compare lines 6 and 7). Since Hfq does not control gcvB transcription, the results suggest that Hfq likely either plays a direct role in GcvB-mediated repression of dppA::lacZ and oppA::phoA or functions in increasing the stability of GcvB.

### Hfq likely affects GcvB–mRNA pairing

Hfq, a general RNA chaperone, has been shown to directly stimulate RNA–RNA pairing (Moller et al., 2002; Zhang et al., 2002) and to stabilize sRNAs (Masse & Gottesman, 2002; Moller et al., 2002; Zhang et al., 2003). We identified a region in the gcvB sequence complementary to both the dppA and oppA mRNAs near their respective ribosome binding sites (Pulvermacher et al., 2008). For dppA, the complementary region is upstream of the translation start site and for oppA the complementary region overlaps the translation start site. This suggests that GcvB–mRNA pairing is a mechanism for GcvB-dependent regulation of dppA and oppA mRNA. Previous mutation analysis of gcvB, dppA and oppA suggests that RNA–RNA pairing is likely part of the mechanism that GcvB uses to control dppA and oppA expression (Pulvermacher et al., 2008). In addition, in Salmonella, GcvB has been shown to base pair with dppA and oppA mRNAs ( Sharma et al., 2007). If the role of Hfq is to facilitate GcvB–mRNA pairing, we predicted that overexpression of GcvB in a WT strain would increase repression of dppA::lacZ. However, overexpression of GcvB in a Δhfq mutant should have little effect on dppA::lacZ expression if Hfq is required for the GcvB–dppA mRNA interaction. Overexpression of GcvB from the multicopy plasmid pGS571 carrying WT gcvB (pgcVB\(^+\)) in the WT ΔdppA::lacZ lysogen resulted in an additional 8.5-fold repression of dppA::lacZ compared with the WT lysogen (Table 3, compare lines 1 and 3). However, overexpression of GcvB in the Δhfq ΔdppA::lacZ [pgcVB\(^+\)] lysogen only resulted in a 1.5-fold repression of dppA::lacZ compared with the Δhfq ΔdppA::lacZ lysogen (Table 3, compare lines 4 and 5). Thus, Hfq is likely needed to facilitate pairing between GcvB and dppA mRNA, and the results are consistent with GcvB and Hfq being part of the same regulatory mechanism. It is possible that the failure of GcvB to repress dppA::lacZ in Δhfq ΔdppA::lacZ [pgcVB\(^+\)] is due in part to decreased stability of the GcvB RNA. Although Hfq does partially stabilize GcvB in WT E. coli (see below), reduced GcvB levels seem an unlikely explanation for loss of repression in Δhfq ΔdppA::lacZ [pgcVB\(^+\)] for the following reasons.

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**Table 2. Effects of Hfq on dppA::lacZ, gcvB::lacZ and oppA::phoA expression**

<table>
<thead>
<tr>
<th>Line</th>
<th>Lysogen*</th>
<th>Relevant genotype</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GS162ΔdppA::lacZ</td>
<td>WT</td>
<td>62 ± 12</td>
</tr>
<tr>
<td>2</td>
<td>GS1132ΔdppA::lacZ</td>
<td>ΔgcvAB</td>
<td>604 ± 3</td>
</tr>
<tr>
<td>3</td>
<td>GS1148ΔdppA::lacZ</td>
<td>Δhfq</td>
<td>994 ± 1</td>
</tr>
<tr>
<td>4</td>
<td>GS1149ΔdppA::lacZ</td>
<td>ΔgcvAB Δhfq</td>
<td>1155 ± 139</td>
</tr>
<tr>
<td>5</td>
<td>GS1149ΔdppA::lacZ [pGS341]</td>
<td>ΔgcvAB Δhfq [pgcVA(^+)]</td>
<td>1383 ± 96</td>
</tr>
<tr>
<td>6</td>
<td>GS162ΔgcvB::lacZ</td>
<td>WT</td>
<td>276 ± 23</td>
</tr>
<tr>
<td>7</td>
<td>GS1148ΔgcvB::lacZ</td>
<td>Δhfq</td>
<td>358 ± 7</td>
</tr>
<tr>
<td>8</td>
<td>GS162ΔoppA::phoA</td>
<td>WT</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>9</td>
<td>GS1132ΔoppA::phoA</td>
<td>ΔgcvAB</td>
<td>77 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>GS1148ΔoppA::phoA</td>
<td>Δhfq</td>
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<td>11</td>
<td>GS1149ΔoppA::phoA</td>
<td>ΔgcvAB Δhfq</td>
<td>71 ± 1</td>
</tr>
<tr>
<td>12</td>
<td>GS1149ΔoppA::phoA [pGS341]</td>
<td>ΔgcvAB Δhfq [pgcVA(^+)]</td>
<td>77 ± 10</td>
</tr>
</tbody>
</table>

*Lysogens were grown in LB, LB + CM or LB + CM + AP to OD\(_{600}\) ~0.5, harvested, and assayed for either β-galactosidase activity (Miller, 1972) or alkaline phosphatase activity (Brickman & Beckwith, 1975).*
Table 3. Effects of overexpression of gcvB on dppA::lacZ expression in an hfq mutant

<table>
<thead>
<tr>
<th>Line</th>
<th>Lysogen*</th>
<th>Relevant genotype</th>
<th>β-Galactosidase activity</th>
<th>Generation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GS162ΔdppA::lacZ</td>
<td>WT</td>
<td>51 ± 1</td>
<td>46 ± 4</td>
</tr>
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<td>2</td>
<td>GS1144ΔdppA::lacZ</td>
<td>ΔgcvB</td>
<td>604 ± 42</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>3</td>
<td>GS162ΔdppA::lacZ [pGS571]</td>
<td>ΔgcvB [pgcvB++]</td>
<td>6 ± 1</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>4</td>
<td>GS1148ΔdppA::lacZ</td>
<td>Δhfq</td>
<td>848 ± 69</td>
<td>63 ± 8</td>
</tr>
<tr>
<td>5</td>
<td>GS1148ΔdppA::lacZ [pGS571]</td>
<td>Δhfq [pgcvB++]</td>
<td>583 ± 20</td>
<td>81 ± 8</td>
</tr>
</tbody>
</table>

*The lysogens and transformants were grown in LB, LB+CM or LB+CM+AP to OD₆₀₀ ~0.5 and assayed for β-galactosidase activity (Miller, 1972).

Northern blot analysis of GcvB expression from WT [pgcvB++], ΔgcvB [pgcvB++] and Δhfq [pgcvB++] strains grown to mid-exponential phase in LB showed roughly equivalent amounts of GcvB produced by the three strains (Fig. 1). Additionally, high levels of GcvB are known to inhibit cell growth (Urbanowski et al., 2000). The presence of extra GcvB in Δhfq ΔdppA::lacZ [pgcvB++] increased the generation time from 63 min without the plasmid to 81 min in the transformed strain (Table 3, compare lines 4 and 5). This suggests that GcvB was expressed in Δhfq ΔdppA::lacZ [pgcvB++] and at levels high enough to begin to inhibit cell growth. These results suggest that the presence of GcvB in a Δhfq strain is unable to restore GcvB repression of the dppA::lacZ fusion.

Effects of Hfq on GcvB stability in LB

Hfq binds RNA with short single-stranded AU-rich sequences preceded or followed by stem–loop structures (Brescia et al., 2003; Moller et al., 2002; Zhang et al., 2003). These sequences are often sites for RNase E degradation (Moll et al., 2003). One effect and therefore likely role of Hfq binding is to increase the stability of sRNAs by preventing degradation by RNase E (Masse & Gottesman, 2002; Moll et al., 2003; Moller et al., 2002; Zhang et al., 2003). It has been reported previously that GcvB is a target for Hfq binding in E. coli (Zhang et al., 2003), and GcvB has several single-stranded AU tracts that could serve as Hfq binding sites (Fig. 2). Thus, we used Northern blots to determine the effect of a Δhfq allele on GcvB stability in E. coli grown in LB. WT, Δhfq or complemented Δhfq [pHFq++] cultures were grown to mid-exponential phase in LB, and rifampicin was added to terminate transcription. GcvB RNA stability was monitored over 10 min (see Methods). For strains grown in LB, GcvB RNA was stable through 10 min in the WT and the complemented Δhfq

Fig. 1. Northern blot analysis of GcvB expression in the WT [pgcvB++] [GS162 (pGS571)], ΔgcvB [pgcvB++] [GS1144 (pGS571)] and Δhfq [pgcvB++] [GS1148 (pGS571)] strains. All strains were grown in LB+AP to OD₆₀₀ ~0.5. Total cell RNA was isolated from each strain, and Northern blot analysis was performed as described in Methods using 5 μg total RNA. Each membrane was first probed with a DIG-labelled DNA probe for GcvB. Blots were then stripped and reprobed using a DIG-labelled DNA probe for 5S RNA. Band intensities were quantified as described in Methods.

Fig. 2. Proposed secondary structure of E. coli GcvB using the mfold prediction program (Zuker, 2003). Predicted stem–loops are lettered A–F and are predicted to be conserved among E. coli, Shigella dysenteriae, Yersinia pestis, Haemophilus influenzae and Vibrio cholerae bacteria. Possible Hfq binding regions are indicated by arrows.
Fig. 3. Northern blot analysis of GcvB stability from (a) WT (GS162), Δhfq (GS1148) and Δhfq [phfq ] [GS1148 (pGS609)] strains and (b) the WT (GS162) strain. All strains were grown in LB to OD600 ~0.5, and rifampicin (250 μg ml−1) was added to stop transcription. Aliquots of cells were taken (a) at 0, 1, 2, 3, 5 and 10 min after the addition of rifampicin or (b) at 0, 2, 10, 20 and 30 min after the addition of rifampicin. Total cell RNA was isolated from each strain at each time point, Northern blot analysis was performed as described in Methods, and each membrane was probed sequentially with DIG-labelled DNA probes specific for GcvB or 5S RNA. The strain from which the RNA was isolated is indicated to the left of each blot and the probe used is indicated after the strain name. The time (in minutes) after the addition of rifampicin is labelled across the top of the blots in (a) and (b). Band intensities were quantified as described in Methods.

Analysis using three different gcvB–lacZ transcriptional fusions indicates that two sizes of GcvB are produced (Urbanowski et al., 2000). These fusions all have a common 5′ end but contain three different fusion points downstream with translationally competent lacZYA. These fusion points occur preceding both predicted terminator sites t1 and t2, between terminators t1 and t2, or after terminator t2 (Urbanowski et al., 2000). Results from these three transcriptional fusions showed that nearly 90 % of the β-galactosidase activity observed with the fusion occurring prior to both terminators t1 and t2 is lost when the fusion point follows terminator t1, indicating that the major site of transcriptional termination is the t1 site. Analysis also found that terminator t2 is also functional, but less GcvB of the 206 nt size is predicted to be produced compared with the 130 nt size. Despite these in vivo transcriptional fusion data indicating that two sizes of GcvB are produced (Urbanowski et al., 2000), we only detected the 206 nt GcvB RNA by Northern blot analysis. Similarly, GcvB in Salmonella also appears to have two potential termination sites, but only one band is detected by Northern analysis, corresponding to a ~200 nt full-length GcvB sRNA (Sharma et al., 2007). Whether the 130 nt RNA in E. coli is too unstable to be detected by Northern blot analysis or whether the terminator 1 site is not functional is currently unclear.

Expression of GcvB in different strains grown in LB

We also determined how much GcvB RNA is produced in various strains grown in LB. A Northern blot was performed using WT, ΔgcvB, ΔgcvB [pgcvB] and Δhfq strains grown in LB to mid-exponential phase. We observed the highest levels of GcvB RNA in either the WT or the complemented ΔgcvB [pgcvB] strain (Fig. 4, lanes 1 and 3). There was approximately threefold less

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (min)</th>
<th>0</th>
<th>2</th>
<th>10</th>
<th>20</th>
<th>30</th>
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Fig. 4. Northern blot comparison of GcvB RNA levels from WT (GS162), ΔgcvB (GS1144), ΔgcvB [pgcvB] (GS1144 (pGS594)) and Δhfq (GS1148) strains grown in LB. An 8% denaturing polyacrylamide gel was run with 5 μg total RNA from each strain. The strain from which the RNA was isolated is indicated at the top of the blot. Blots were probed with DIG-labelled DNA probes specific for GcvB or 5S RNA. Band intensities were quantified as described in Methods.
GcvB in the Δhfq strain compared with the WT and the complemented ΔgcvB [pgcvB +] strains, confirming that a function of Hfq is to stabilize GcvB RNA (Fig. 4, compare lane 4 with lanes 1 and 3). As expected, no GcvB RNA was detected from the ΔgcvB strain (Fig. 4, lane 2). The ability to detect significant levels of GcvB in the Δhfq strain, and the observation that there is a loss of regulation of both dppA:: lacZ and oppA:: phoA in the Δhfq strain (Table 2) support the hypothesis that Hfq has a function in addition to stabilizing GcvB. Previous results (Pulvermacher et al., 2008), as well as studies in Salmonella (Sharma et al., 2007), suggest that this additional function is to facilitate GcvB–target mRNA pairing.

Expression of GcvB in WT cells grown in different media

Earlier analysis of the GcvB sRNA in Salmonella has found that GcvB is detectable only in LB and not in minimal media (Sharma et al., 2007). To determine whether GcvB is expressed similarly in E. coli we performed a Northern blot comparing GcvB expression among WT cells in the mid-exponential phase of growth grown in LB, GM, GM + glycine and GM + inosine media. WT cells grown in LB produced the most GcvB with approximately fourfold less GcvB detected in GM + glycine-grown cells (Fig. 5, compare lanes 1 and 2). We did not observe GcvB when WT cells were grown in GM or GM + inosine (Fig. 5, lanes 3 and 4). This result is not entirely surprising, since gcvB is activated by the GcvA protein, and GcvA is unable to fully activate gcvB expression in GM and GM + inosine media (Urbanowski et al., 2000). This inability to detect GcvB RNA in GM and GM + inosine media may explain why GcvB was not detected when WT Salmonella was grown in minimal media lacking glycine (Sharma et al., 2007). We hypothesize that GcvB regulates other genes in defined media in conjunction with the glycine cleavage system. If true, these target genes likely respond to lower levels of GcvB than dppA and oppA.

Effects of Hfq on GcvB stability in GM+glycine

Negative regulation of dppA:: lacZ and oppA:: phoA occurs in LB, but not in GM + glycine-grown cells (Urbanowski et al., 2000). However, we observed approximately eightfold higher β-galactosidase levels from a WT ΔgcvB:: lacZ lysogen grown in GM + glycine compared with GM and ~25-fold higher levels in GM + glycine compared with GM + inosine (Urbanowski et al., 2000), suggesting that GcvB is made and has regulatory targets other than dppA and oppA in GM + glycine. We have shown that GcvB is detectable in GM + glycine media. Therefore, we analysed the stability of GcvB from cells grown in GM + glycine to determine whether Hfq also functions to stabilize GcvB in defined media. When WT, Δhfq and complemented Δhfq [phfq +] strains were grown in GM + glycine, we determined the half-life of GcvB RNA to be ~3 min in WT and complemented Δhfq [phfq +] cells, while the half-life of GcvB in Δhfq was less than 1 min (Fig. 6). Thus, the half-life of GcvB in WT and Δhfq cells is much lower in GM + glycine compared with that of the same strains grown in LB. This result likely explains why we did not observe negative regulation of dppA:: lacZ or oppA:: phoA in GM + glycine, as the GcvB levels may be too low to allow normal regulation. However, it is interesting that we were

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Fig. 5. Northern blot comparison of GcvB RNA levels from different media. An 8% denaturing polyacrylamide gel was run with the following total RNA: lane 1, 5 µg total RNA isolated from WT (GS162) cells grown in LB; lanes 2–4, 10 µg total RNA isolated from WT (GS162) cells grown in GM + glycine, GM and GM + inosine, respectively. The strain from which the RNA was isolated is shown at the top of the blot, and the respective medium in which each strain was grown is given below the blot. The blot was probed using DIG-labelled DNA probes specific for GcvB or 5S RNA. Band intensities were quantified as described in Methods.

Fig. 6. Northern blot analysis of GcvB stability from WT (GS162), Δhfq (GS1148) and Δhfq [phfq +] [GS1148 (pGS609)] strains. All strains were grown in GM + glycine to OD600 ~0.5, and rifampicin (250 µg ml−1) was added to stop transcription. Aliquots of cells were taken at 0, 1, 2, 3, 5 and 10 min after the addition of rifampicin. Total cell RNA was isolated from each strain at each time point, Northern blot analysis was performed as described in Methods, and each membrane was probed with a DIG-labelled DNA probe specific for either GcvB or 5S RNA. The strain from which the RNA was isolated is indicated at the left along with the respective probe used. The time (in minutes) after the addition of rifampicin is indicated across the top. Band intensities were quantified as described in Methods.
able to show that GcvB is produced in both WT and Δhfq cells grown in GM + glycine. Based on our results, Hfq functions to stabilize GcvB in cells grown in both LB and GM + glycine.

Timing of GcvB synthesis

We determined when GcvB is expressed in cells to help determine potential regulatory targets of GcvB. WT cells were grown in either LB or GM + glycine medium, RNA was harvested from various stages of growth, and Northern blots were performed. In LB-grown cells, GcvB RNA was detected from early exponential phase through early stationary phase (Fig. 7a, lanes 1–5), with the highest levels detected at the mid-exponential phase (Fig. 7a, lane 2). *Salmonella* GcvB is also detected only from cells grown to exponential phase in LB (Sharma *et al.*, 2007). When WT *E. coli* cells were grown in GM + glycine, a similar GcvB expression pattern was observed, with GcvB RNA detected from early exponential phase through early stationary phase (Fig. 7b, lanes 1–4). No GcvB was detected under either growth condition when RNA was isolated from cells that had entered stationary phase (Fig. 7a, lane 6; Fig. 7b, lanes 5 and 6). The results suggest that GcvB regulates targets only during the exponential and early stationary phases of growth. Additionally, the level of Hfq has been shown to decrease gradually during the transition from exponential phase to stationary phase (Ali Azam *et al.*, 1999). These findings suggest that Hfq is maintained at a higher level during the exponential phase because it plays a role in regulation of targets during this growth stage. It will be interesting to determine whether GcvB has additional regulatory targets in the exponential phase in LB- or GM + glycine-grown cells and whether all GcvB regulatory targets are dependent upon Hfq for regulation.

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REFERENCES


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