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# *Clostridium difficile* Hfq can replace *Escherichia coli* Hfq for most of its function

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## ABSTRACT

A gene for the Hfq protein is present in the majority of sequenced bacterial genomes. Its characteristic hexameric ring-like core structure is formed by the highly conserved N-terminal regions. In contrast, the C-terminal forms an extension, which varies in length, lacks homology, and is predicted to be unstructured. In Gram-negative bacteria, Hfq facilitates the pairing of sRNAs with their mRNA target and thus affects gene expression, either positively or negatively, and modulates sRNA degradation. In Gram-positive bacteria, its role is still poorly characterized. Numerous sRNAs have been detected in many Gram-positive bacteria, but it is not yet known whether these sRNAs act in association with Hfq. Compared with all other Hfqs, the *C. difficile* Hfq exhibits an unusual C-terminal sequence with 75% asparagine and glutamine residues, while the N-terminal core part is more conserved. To gain insight into the functionality of the *C. difficile* Hfq (Cd-Hfq) protein in processes regulated by sRNAs, we have tested the ability of Cd-Hfq to fulfill the functions of the *E. coli* Hfq (Ec-Hfq) by examining various functions associated with Hfq in both positive and negative controls of gene expression. We found that Cd-Hfq substitutes for most but not all of the tested functions of the Ec-Hfq protein. We also investigated the role of the C-terminal part of the Hfq proteins. We found that the C-terminal part of both Ec-Hfq and Cd-Hfq is not essential but contributes to some functions of both the *E. coli* and *C. difficile* chaperons.

**Keywords:** Hfq; *Escherichia coli*; *Clostridium difficile*; small noncoding RNAs; gene expression

## INTRODUCTION

Hfq is a small protein (102 amino acid residues in *Escherichia coli*) encoded by the *hfq* gene. Hfq belongs to an ancient family of RNA-binding proteins that is implicated in RNA-mediated reactions in all three domains of life and plays a pivotal role in the control of gene expression (for a recent review, see Vogel and Luisi 2011). Eukaryotic and archaeal homologs of Hfq are, respectively, Sm-proteins and the closely related Sm-like (LSm) proteins. Sm proteins are arranged in a multimeric ring-like quaternary structure comprising seven different monomers. In contrast, the bacterial Hfq proteins form homohexameric rings. Hfq is a regulatory protein that has recently received much attention because of its crucial role in cellular processes controlled by small noncoding RNAs (sRNAs). Hfq facilitates the pairing of sRNAs with their target mRNAs, thereby affecting their expression either positively or negatively. Moreover, Hfq plays an important role in modulating mRNA degradation and RNA transcription (Folichon et al. 2003; Le Derout et al. 2010). Hfq is present in half of the bacterial sequenced genomes including many

pathogens (Chao and Vogel 2010). Hfq was shown to act as a virulence factor in several bacterial pathogens (for review, see Hajnsdorf and Boni 2012), but it is not clear whether it can be considered as a general factor required for the virulence of all Hfq-containing pathogens.

The *E. coli* and *Staphylococcus aureus* Hfqs have been crystallized. The ring-like structure (the so-called core made up of two Sm motifs), is formed from the highly conserved N-terminal parts of Hfq molecule (aa 1–65 in *E. coli*). A number of amino acids in the *E. coli* protein have been identified as important for interaction with RNA. Three RNA-binding sites were defined by groups of residues and called the proximal (Q8, D9, F39, Y55, K56, F42), distal (Y25, I30), and the rim (R16, R19, and R17) RNA-binding surfaces (Mikulecky et al. 2004; Zhang et al. 2013). While the core of the various Hfq paralogs is rather conserved in sequence and structure (residues 7–66 in *E. coli*) (Fig. 1), in contrast, the C-terminal sequences have different lengths and lack homology in different species (Fig. 1). Hfq proteins of  $\gamma$ - and  $\beta$ -proteobacteria have an extended C terminus (up to 38, mainly hydrophilic,

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**FIGURE 1.** Multiple sequence alignment of Hfq from different species. The ortholog cluster multiple alignment of the amino acids sequences of Hfq proteins of several Clostridia and various Gram-positive or Gram-negative model species was performed by using ClustalW on the website <http://mbgd.genome.ad.jp>. Proximal face residues important for the interaction with RNA are highlighted in gray, those important for distal face interaction and at the rim are double underlined and underlined, respectively, both in the Ec-Hfq (Mikulecky et al. 2004; Zhang et al. 2013) and Cd-Hfq. The numbering is based on Ec-Hfq. The secondary structure is indicated by boxes ( $\alpha$ -helix) and arrows ( $\beta$  sheets). The Sm motifs are boxed with dotted lines. The core structured part of Ec-Hfq and Cd-Hfq are boxed with plain lines.

amino acids in *E. coli* and close relatives), whereas some Gram-positive bacteria, including *Bacillus subtilis* and *Staphylococcus aureus*, have Hfq proteins with short (<10 amino acid) C-terminal extensions (Sauter et al. 2003). The role of the C-terminal part has not been elucidated. In *E. coli*, it was shown that the *hfq2:: $\Omega$*  mutation, which leaves 79 N-terminal amino acids intact out of 102 residues, does not affect its function and had no obvious phenotype (Tsui and Winkler 1994). The C-terminal domain of *E. coli* Hfq is dispensable for hexamer formation, a truncated form of *E. coli* Hfq deprived of its 19 C-terminal residues is fully able to bind to polyadenylated *rpsO* mRNA (Arluison et al. 2004) and sRNA DsrA (Sonnleitner et al. 2004). But more recent data show that the C-terminal domain is required for binding to long mRNAs like *rpoS* (Vecerek et al. 2008; Beich-Frandsen et al. 2011) and to activate GlmS expression by GlmY but not GlmZ (Salim et al. 2012). The C-terminal part possesses no identifiable motif, it is flexible and in the crystallographic structure extends laterally away from the hexameric core and exhibits features typical of intrinsically disordered proteins. Some intrinsically disordered domains have been implicated in facilitating intermolecular interactions (Babu et al. 2011). It has been suggested that a disordered C-terminal

domain could participate in the interaction of Hfq with RNA molecules (Beich-Frandsen et al. 2011).

In Gram-negative bacteria, it is well-established that Hfq assists sRNAs in their regulatory function, while in Gram-positive bacteria, its role is still poorly characterized (Jousselin et al. 2009; Waters and Storz 2009; Chao and Vogel 2010). Although numerous sRNAs have been detected in many Gram-positive bacteria, it is not yet clear whether Hfq is implicated in sRNA network. In *Listeria monocytogenes*, Hfq has been shown to play a role in stress tolerance and virulence (Christiansen et al. 2004) and its inactivation does not generally affect sRNAs abundance, only three sRNAs coimmunoprecipitated with Hfq (Christiansen et al. 2006; Mandin et al. 2007; Toledo-Arana et al. 2009) with facilitation of LhrA binding to its targets (Nielsen et al. 2010, 2011). In *S. aureus*, Hfq is neither required for RNAIII function nor for sRNA stabilization (Boisset et al. 2007; Geissmann et al. 2009).

The role of Hfq homologs from several bacteria has been previously tested by complementation of *hfq* mutations in *E. coli*. These studies revealed that heterologous *hfq* genes may or may not reverse phenotypes associated with *hfq* deficiency in *E. coli*. As examples, *Pseudomonas aeruginosa* Hfq, which comprises only 82 amino acids, and the *Moraxella catarrhalis hfq* gene (210 amino acids) both functionally replace the *E. coli* protein (Sonnleitner et al. 2002; Attia et al. 2008), but the Hfq homologs of *Synechocystis* sp.PCC 6803 and *Anabaena* PCC 7120 (Boggild et al. 2009) are not able to replace *E. coli* Hfq while *Staphylococcus aureus* Hfq fails to substitute for *Salmonella typhimurium* Hfq (Rochat et al. 2012). However, the experimental conditions greatly varied in these studies regarding the expression system of the Hfq homologs as well as the functions examined.

An increasing amount of data shows that the Clostridia also contain sRNAs (Chen et al. 2011; Mraheil et al. 2011). For example, a great number and a large diversity of regulatory RNAs have been recently identified in the pathogenic clostridium *Clostridium difficile* (Soutourina et al. 2013). Moreover, a gene (*CD1974*), homologous to *hfq*, has been shown to be transcribed in this bacterium (I Verstraete, O Soutourina, pers. comm.). The *C. difficile* Hfq (Cd-Hfq) protein exhibits 46% identity (31 amino acids out of the first 66 amino acids) with Ec-Hfq. Most of the key residues that have been identified as important for interaction with RNA in the *E. coli* Hfq protein are present in Cd-Hfq: e.g., in the

proximal binding surface (Q8, D9, F39, Y55, K56, but not F42 which is replaced by Y, also an aromatic residue), in the distal site (Y25, I30) and at the rim (R16, R19, but not R17, replaced by K and which retains the positive charge) (Mikulecky et al. 2004; Zhang et al. 2013). However, the Cd-Hfq C-terminal domain is very different; it is much shorter (16 aa) and includes an unusual stretch of seven asparagine residues, unique to this species (Fig. 1).

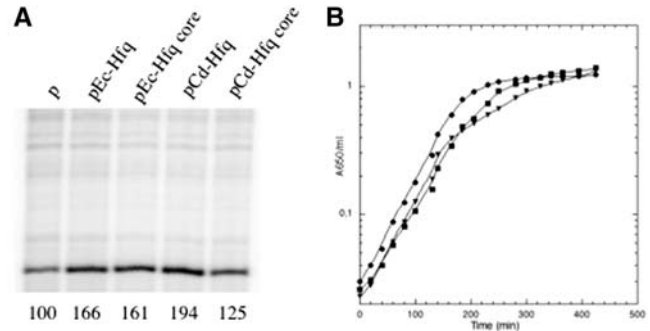
To investigate the functionality of the Cd-Hfq protein and its potential role in sRNA regulation of gene expression, we tested the ability of Cd-Hfq to complement various phenotypes associated with the *E. coli* *hfq* mutation. We have examined four specific functions of Hfq where the involvement of Ec-Hfq has previously been well-documented. We show that Cd-Hfq is as efficient as Ec-Hfq in the negative control of OppA expression and in the positive control of RpoS expression but with differential effects on the stability of the sRNAs involved. In addition, Cd-Hfq is as proficient as Ec-Hfq in controlling Ec-Hfq synthesis. Surprisingly, Cd-Hfq does not participate in the negative control of PtsG carried out by SgrS. In addition we show that deletion of the C-terminal extensions despite having different primary sequences similarly impact the regulatory function of the two proteins.

## RESULTS

### Cd-Hfq is expressed at similar levels as Ec-Hfq in *E. coli*

The Cd-*hfq* ORF was cloned in place of the Ec-*hfq* open reading frame of plasmid pTX381 (a low copy-number derivative of pACYC184) (Tsui et al. 1994). These two plasmids are hereafter designated as pCd-Hfq and pEc-Hfq, respectively. We also cloned the two core proteins without the C-terminal part: pEc-Hfq core and pCd-Hfq core, corresponding to the 65 and 68 N-terminal amino acids of the two proteins, respectively (Fig. 1).

We first verified that the four Hfq proteins were expressed at comparable levels from the plasmids. All *hfq* variants are expressed from both P<sub>2hfq</sub> and P<sub>3hfq</sub> promoters present on the original pTX381 plasmid (Tsui et al. 1994, 1996, 1997). The relative expression levels of the different *hfq* variants was estimated by performing an abortive primer extension with an oligonucleotide that hybridizes to the 5' UTR of the *hfq* gene present in all the constructs. This sequence is still present in the chromosomal *hfq* deletion and also in the *hfq-lacZ* chromosomal fusion in strain IBhfq95  $\Delta hfq$  (Fig. 2A, lane p; Ziolkowska et al. 2006). The reverse transcript measured in the strain carrying the vector plasmid corresponds to these two transcripts (Fig. 2A). The presence of the plasmids expressing full-size and core Hfqs increases the amounts of the *hfq* UTR transcript less than twofold, showing that *hfq* is expressed from the plasmids at levels comparable to the physiological levels.



**FIGURE 2.** Complementation by Hfq variants of growth phenotype and expression of Hfq. (A) Total RNA from strains IBhfq95  $\Delta hfq$  transformed with the empty vector (p), pEc-Hfq, pEc-Hfq core, pCd-Hfq, or pCd-Hfq core were used as templates for reverse transcriptase primed with *hfq* primer, which hybridizes upstream of the *hfq* ORF, and carried out in abortive conditions. Quantification of the radioactivity (arbitrary units) was indicated below the gel. The transcript detected in the plasmid vector containing strain (IBhfq95  $\Delta hfq/p$ ) corresponds to the 5' end of mRNA covering the *hfq* deletion on the chromosome and the 5' end of the *hfq-lacZ* fusion present at *lacZ* (Ziolkowska et al. 2006); both should respond to Hfq autoregulation. Fainter bands detected on the same gel due to unspecific hybridization of the primer do not vary between samples, revealing that equivalent loading has been applied on the gel. (B) Wild-type strain carrying the empty vector (p) ( $\blacktriangledown$ ) and the  $\Delta hfq$  mutant transformed with pEc-Hfq ( $\bullet$ ) and pCd-Hfq ( $\blacksquare$ ) were grown in LB medium with appropriate antibiotics at 37°C. Turbidity of the cultures was monitored at 37°C.

An Hfq mutant exhibits several phenotypes compared to the wt strain. In LB liquid medium the growth of the mutant is diauxic with a decrease of growth rate and an entry into stationary phase at a lower OD than for the wt (Tsui et al. 1994). We first compared growth in LB medium and found that the *hfq* mutant transformed with pCd-Hfq exhibited a growth intermediary between the control strain ( $\Delta hfq/p$ ) and the complemented strain ( $\Delta hfq/pEc-Hfq$ ) (Fig. 2B), giving the first indication that the Cd-*hfq* gene is functional in *E. coli*. Removing the C-terminal tails had only a minimal effect on the complementation efficiency, suggesting that the C-terminal tail of Ec as well as Cd is probably dispensable in the tested conditions.

### Cd-Hfq is functional for Hfq autoregulation in *E. coli*

To further compare the Cd-Hfq and Ec-Hfq proteins, we examined their ability to negatively affect the expression of an *hfq::lacZ* fusion under the control of the P<sub>3hfq</sub> promoter. Hfq autoregulates its own expression fourfold by binding to its own mRNA, although the mechanism has not been completely elucidated (Vecerek et al. 2005; Ziolkowska et al. 2006). Table 1 shows that Ec-Hfq and Cd-Hfq exhibit the same repression factor (4.0) but that the plasmids expressing just the N-terminal cores of Ec-Hfq and Cd-Hfq were inactive in repression. This implies that the C-terminal unstructured region of the two proteins might be important for their interaction with the 5' UTR of the *hfq* transcript or for the stability of the proteins.



**TABLE 1.** Cd-Hfq can substitute for Ec-Hfq to regulate the expression of *hfq-lacZ*, but the C-terminal domain of the two proteins is required

Hfq	$\beta$ -Galactosidase activity	Repression factor
None	41.3 $\pm$ 3.3	–
Ec-Hfq	10.2 $\pm$ 0.5	4.0
Ec-Hfq core	30.7 $\pm$ 0.8	1.3
Cd-Hfq	10.3 $\pm$ 0	4.0
Cd-Hfq core	43.3 $\pm$ 0.3	1.0

IBhfq95  $\Delta$ *hfq* strain carrying an *hfq-lacZ* fusion on the chromosome was transformed with the empty vector (p), pEc-*hfq*, pEc-Hfq core, pCd-*hfq*, and pCd-*hfq* core.  $\beta$ -galactosidase was measured in bacteria harvested in exponential phase. Mean values are calculated from three independent experiments.

### Negative regulation of *oppA* expression by Cd-Hfq

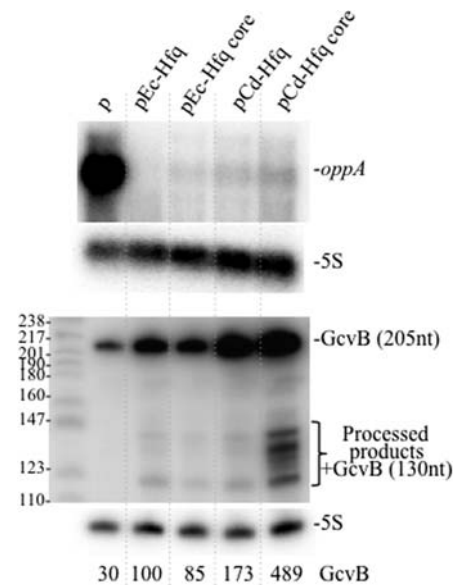
A 56-kDa protein identified as OppA, a periplasmic component of the oligopeptide transport system, was detected only in the *hfq* mutant (Ziolkowska et al. 2006). We took advantage of this easy assay to examine the effect of the various Hfq derivatives on *oppA* expression. Coomassie blue staining did not reveal any detectable band corresponding to OppA protein in the strains containing *hfq* genes expressed from the plasmid (data not shown). This result was confirmed by the absence of *oppA* mRNA transcript in the same strains indicating that all the Hfq proteins tested inhibit the synthesis of this polypeptide (Fig. 3). OppA repression is achieved by Hfq and GcvB, a conserved sRNA, which targets many ABC transporters of small peptides, amino acids, and also proteins involved in amino acid biosynthesis pathways (Sharma et al. 2007, 2011). GcvB is an Hfq-dependent sRNA, its transcription is independent on Hfq and it is unstable in the *hfq* mutant (Urban and Vogel 2007; Pulvermacher et al. 2009). We analyzed its level by Northern blot and found that the expression of Ec-Hfq increases its level threefold compared with the *hfq* mutant, and only a slightly reduced level was found in the strain with the deletion of the C terminus domain (Fig. 3). Surprisingly, GcvB levels were higher when the Cd-Hfq protein, with or without the C-terminal region, was expressed from the plasmid. Moreover, a 130-nt processed GcvB form, already observed by Urbanowski et al. (2000), and other degradation products were detected only when the Cd-Hfq core was present (Fig. 3). We conclude that Cd-Hfq is as efficient as the *E. coli* protein for the negative control of OppA expression and that the C-terminal domains of Ec-Hfq and Cd-Hfq are not necessary for this regulation. However, Cd-Hfq has a greater stabilizing effect on GcvB sRNA than Ec-Hfq with a role of the Cd-Hfq C-terminal domain.

### Cd-Hfq activates *rpoS* expression

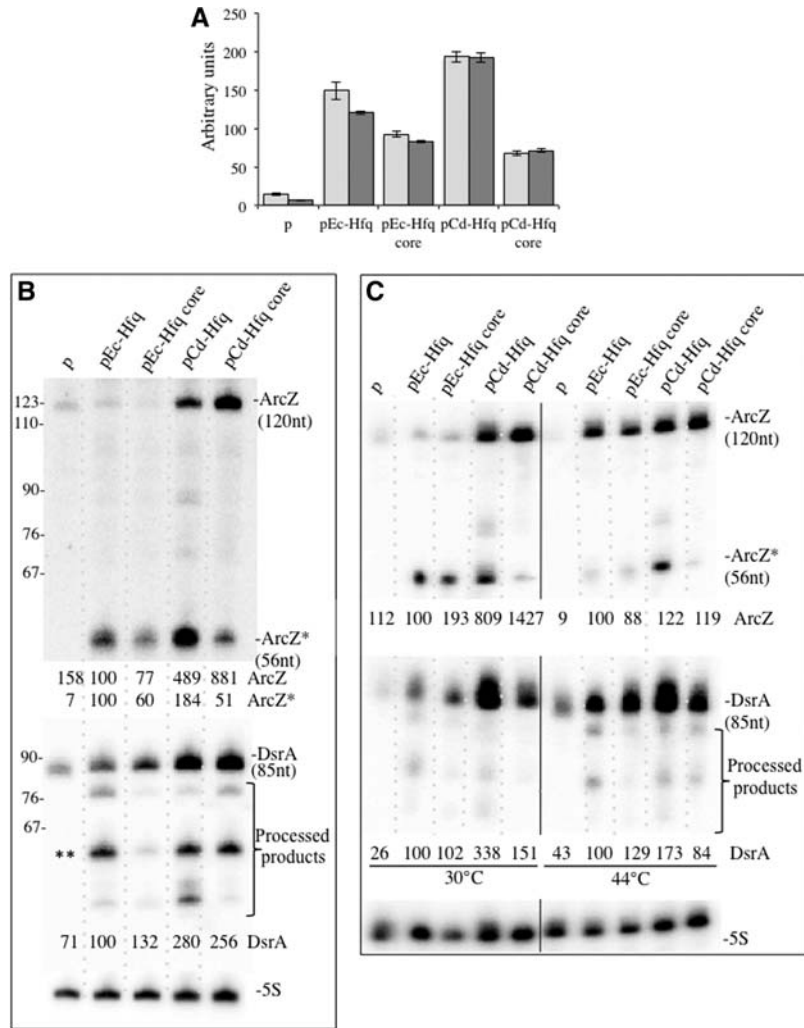
An increase in RpoS expression occurs under the conditions of nutrient deprivation, stress or at the entry into the stationary phase. This accumulation is regulated at multiple

levels. Three sRNAs DsrA (Majdalani et al. 1998), RprA (Majdalani et al. 2001), and ArcZ (Mandin and Gottesman 2010) activate the translation of RpoS while OxyS represses it (Zhang et al. 1998), and all of these regulatory processes are Hfq-dependent. Therefore, *rpoS* appears to be an ideal target to examine the regulatory function of the various Hfq constructs. We used a translational *rpoS-lacZ* chromosomal fusion (Ziolkowska et al. 2006) and determined its expression in exponential growing bacteria in the presence of the various *hfq*-expressing plasmids (compared with the plasmid without an Hfq insert). Figure 4A shows that both Ec-Hfq and Cd-Hfq activate the expression of the fusion protein to comparable levels. Deletion of the C-terminal coding sequence of Ec-Hfq and Cd-Hfq partly reduces expression of the fusion, confirming that it is required for optimal regulation of *rpoS* expression (Vecerek et al. 2008; Beich-Frandsen et al. 2011). This latter result indicates that the sequence of the C-terminal extension is not crucial by itself.

The various sRNAs that control the expression of RpoS are expressed under different stress conditions, notably in entrance to stationary phase or nutrient stress. We first concentrated on examining ArcZ since it is expressed in rich medium in exponential growth corresponding to our growth conditions and contributes to *rpoS* expression (Mandin and Gottesman 2010). Hfq coimmunoprecipitates with the ArcZ sRNA (Wassarman et al. 2001; Zhang et al. 2003) and ArcZ is required for RpoS activity (Papenfert et al. 2009). ArcZ (previously named as RyhA/SraH) is transcribed as a 121-nt RNA, which is subsequently processed to generate a



**FIGURE 3.** Cd-Hfq represses *oppA* expression. Northern blots showing the *oppA* transcript and GcvB sRNA detected in strain IBhfq95  $\Delta$ *hfq* transformed with the empty vector (p), pEc-Hfq, pEc-Hfq core, pCd-Hfq, and pCd-Hfq core. Membranes were reprobbed for 5S rRNA to normalize the RNA content. Relative SgrS levels after normalization are indicated below the Northern blot.



**FIGURE 4.** Complementation by Hfq variants of *rpoS* expression. (A) RO91- $\Delta$ *hfq* (light-gray) and RO91- $\Delta$ *hfq arcZ* mutant (dark-gray) were transformed with pACYC184 derivative plasmid expressing either wild-type or C-terminally truncated Hfq proteins from *E. coli* and *C. difficile* giving pEc-Hfq, pEc-Hfq core, pCd-Hfq, and pCd-Hfq core, respectively. The empty vector was used as a control (p).  $\beta$ -galactosidase activity from the *rpoS-lacZ* chromosomal fusion was measured in exponentially growing cells at 37°C (OD<sub>650</sub> of 0.4). Each value is the mean of three independent experiments, with standard deviations not exceeding 15% of magnitude. (B) Northern blots showing ArcZ, ArcZ\*, and DsrA in RO91- $\Delta$ *hfq* transformed with the same plasmids (as in Fig. 3). (\*\*\*) A 61-nt long DsrA processed fragment identified in Repoila and Gottesman (2001). (C) Northern blots showing ArcZ, ArcZ\*, and DsrA in IBPC 928 transformed with the same plasmids, grown at 30°C, and shifted for 15 min to 44°C to inactivate thermosensitive RNase E. Relative amounts of ArcZ and DsrA normalized to 5S RNA are indicated.

smaller, stable RNA that consists of the last 56 nt of the original transcript (nt 66–121 of the full-length transcript, hereafter designated as ArcZ\*) (Argaman et al. 2001; Papenfort et al. 2009; Mandin and Gottesman 2010), which base pairs within the *rpoS* mRNA leader (Mandin and Gottesman 2010). ArcZ\* sRNA is abundant, while the full-length transcript is hardly detected in *E. coli* and *S. typhimurium* and the 5' part is not detectable (Papenfort et al. 2009). Neither the full-length transcript nor the 5' processed region were detected in *S. typhimurium hfq* mutant (Papenfort et al. 2009). Figure 4B shows that the full-length ArcZ sRNA is hardly

*rpoS* in the presence of the different Hfq variants (Fig. 4A). This is not surprising since *rpoS* transcription is very low in our experimental conditions and therefore its expression may be controlled by regulators that are functionally redundant. For this reason we then examined DsrA sRNA, which also contributes to *rpoS* expression in LB medium at 37°C (Mandin and Gottesman 2010) and is unstable in the *hfq* mutant when expressed from the chromosome (Sledjeski et al. 2001). DsrA is more abundant in Ec-Hfq complemented strains than in the mutant but as ArcZ, it is even more abundant in Cd-Hfq complemented strains (Fig. 4B). Both RNase

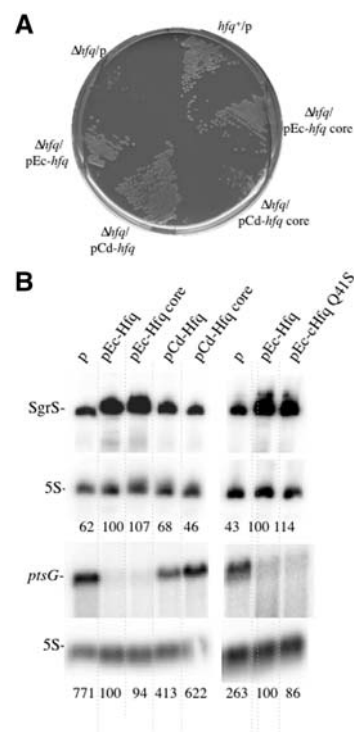
detectable in the *hfq* mutant and in strains complemented by Ec-Hfq but accumulated in the Cd-Hfq complemented strains. Moreover, both the total amount of the two transcripts and their relative abundance varied depending on the origin of the Hfq protein. Since Hfq deficiency does not impair ArcZ transcription (Papenfort et al. 2009), our results suggest that the origin of the Hfq protein differently affected ArcZ stability and/or its processing into ArcZ\*. ArcZ\* levels are high in strains growing in early exponential phase complemented with either Cd-Hfq or Ec-Hfq. In both cases, higher levels of ArcZ\* were detected in the presence of the full-length Hfq compared with the truncated form. Interestingly, the full-length sRNA was more abundant in the presence of Cd-Hfq and Cd-Hfq core. RNase E has been proposed to process the ArcZ sRNA (Papenfort et al. 2009). One interpretation of our results is that Cd-Hfq might be less efficient than Ec-Hfq to recruit RNase E. According to this hypothesis, thermal inactivation of RNase E increases the level of ArcZ and decreases that of ArcZ\* in the presence of Ec-Hfq whereas there is no effect on the levels of both ArcZ and ArcZ\* in the presence of Cd-Hfq (Fig. 4C). However, we cannot exclude that Cd-Hfq strongly interacts with ArcZ in the cell, thus decreasing its turnover. We conclude that Cd-Hfq participates in both RpoS synthesis and ArcZ sRNA stabilization, which are required for the activation of RpoS expression. In Ec as in Cd, the C-term domain has a minor role but could maximize the effects by favoring contact with *rpoS* mRNA as previously reported (Vecerek et al. 2008; Beich-Frandsen et al. 2011). Inactivation of ArcZ has only a faint impact on the expression of

E and RNase III have been shown to cleave DsrA and DsrA-*rpoS* duplex, respectively (Moll et al. 2003; Resch et al. 2008). The number of processed forms we detected is higher than that previously reported and their relative abundance greatly varies making it difficult to establish a link between these processing and the Hfq variant acting in the bacteria. As for GcvB and ArcZ, thermal inactivation of RNase E has no effect on the levels of these processing products while it stabilizes DsrA in Ec-Hfq but not Cd-Hfq containing cells (Fig. 4C), reinforcing our assumption that RNase E cannot interact with Cd-Hfq.

### Cd-Hfq does not trigger *ptsG* control by SgrS

The addition of  $\alpha$ -methylglucoside ( $\alpha$ MG) to wild-type cells rapidly generates phosphosugar stress, resulting in induction of the SgrS sRNA (Vanderpool and Gottesman 2004), which in turn leads to destabilization of *ptsG* mRNA. *PtsG* mRNA degradation is dependent upon RNase E and Hfq in an RNase E-Hfq-SgrS ribonucleoprotein complex (Morita et al. 2003, 2004, 2006). As a consequence, an *hfq* mutant is unable to initiate the phosphostress response and does not grow on plates containing  $\alpha$ MG (Fig. 5A). (Vanderpool 2007). The pCd-Hfq protein as well as the Ec-Hfq and Ec-Hfq core proteins were able to restore growth on  $\alpha$ MG containing plates but not the Cd-Hfq core carrying strain (Fig. 5A). This indicates that Cd-Hfq deprived of its C-terminal domain cannot fully replace Ec-Hfq, and cannot properly regulate *ptsG* expression.

However, strains containing Ec-Hfq and Ec-Hfq core grew more rapidly in liquid LB medium containing  $\alpha$ MG (doubling time of 33 and 35 min, respectively), than strains with Cd-Hfq and Cd-Hfq core (generation time of 68 and 77 min, respectively) (data not shown). The addition of  $\alpha$ MG for 15 min in liquid LB medium caused an increased synthesis of SgrS sRNA in the Ec-Hfq complemented strains compared with the control and pCd-Hfq-containing strains (Fig. 5B). Interestingly, *ptsG* mRNA degradation, which takes place in the strain complemented with Ec-Hfq as previously observed (Morita et al. 2005) and with Ec-Hfq core, is strongly reduced or absent in strains containing the Cd-Hfq proteins (Fig. 5B). In addition, we detected in all cases an inverse correlation between *ptsG* mRNA and SgrS levels. It has previously been postulated that the folded secondary structure of SgrS sRNA has to be modified by Hfq before it can anneal with the *ptsG* mRNA (Maki et al. 2010). The lower SgrS levels detected in strains containing Cd-Hfq constructs might mean that the *C. difficile* proteins inefficiently interact with SgrS sRNA in vivo and as a consequence do not provide the formation of the SgrS-Hfq-RNase E ribonucleic complex required for *ptsG* degradation in the presence of  $\alpha$ MG. Our assumption on the inability of Cd-Hfq in binding to RNase E would strengthen this effect. In addition, our results indicate that for the *E. coli* Hfq, the C-terminal domain is not required for interaction with SgrS and/or *ptsG* mRNA. *E.*



**FIGURE 5.** Cd-Hfq cannot substitute for Ec-Hfq in the negative control of *ptsG* expression. (A) RO91- $\Delta$ *hfq* bacteria transformed with the various plasmids were grown at 37°C on LB plates containing appropriate antibiotics and 0.2%  $\alpha$ -methylglucoside. (B) Northern blots showing SgrS and *ptsG* transcripts detected in strains grown in LB after addition of 1%  $\alpha$ -MG for 20 min. The strain was also transformed with pEc-HfqQ41S.

*coli* Hfq core protein is equally efficient as the full-length Hfq at controlling the expression of *ptsG* by SgrS and at promoting the degradation of the *ptsG* messenger carried out by RNase E.

### Comparison of Ec-Hfq and Cd-Hfq RNA-binding capacity

The RNA-binding capacity of purified His-tagged Ec-Hfq and Cd-Hfq was evaluated in parallel by mobility-shift assay using SgrS, ArcZ, ArcZ\*, and GcvB sRNAs and also the 5'-*hfq* UTR. Determination of the binding constants for half saturation ( $K_{1/2}$ ) shows that Cd-Hfq binds all RNAs but with a 13- to 240-fold higher  $K_{1/2}$  than Ec-Hfq (Table 2). This may be related to a difference in protein stability and/or hexamer formation of the two purified proteins. The two proteins were purified in parallel with similar yields but unlike the Ec-Hfq protein which, immediately after purification, was 50% recalcitrant to denaturation on an SDS gel, there was little hexamer (15%) detected with Cd-Hfq, (Fig. 6). There seemed to be no direct correlation between sRNA abundance in vivo and in vitro binding affinities for Cd-Hfq.

The ortholog cluster multiple alignment of the Hfq proteins of several *Clostridia* and various Gram-positive or



**TABLE 2.** Relative interaction of Ec-Hfq and Cd-Hfq with sRNAs and 5'-UTR *hfq*

RNA	Ec-Hfq (nM)	Cd-Hfq (nM)
GcvB	0.06	12.66
SgrS	0.53	7.2
AcrZ	0.47	10.61
ArcZ*	2	28.9
5'UTR- <i>hfq</i>	0.18	42.89

5' end-labeled RNA indicated at the top of each column was mixed with increasing concentrations of Hfq ranging from 5 pM to 100 nM. Complexes were separated on native polyacrylamide gels (data not shown). The data were plotted using KALEIDAGRAPH 3.0.4 (Abelbeck Software) and the generated curves were fitted to Hill plot, the  $K_{1/2}$  values representing the protein concentration at half-maximal RNA binding. The dissociation constants for Hfq binding to the 4 sRNAs were determined as the  $K_{1/2.5}$  (half saturation values), which were derived from the best fit of the data.

Gram-negative model species (<http://mbgd.genome.ad.jp>) shows that there is an extra amino acid at the Sm1–Sm2 junction only in Cd-Hfq (NTV in Ec-Hfq and DNRQ in Cd-Hfq) (Fig. 1). The model of Cd-Hfq protein based on the known structure of Ec-Hfq (Protein Data Bank entry 1HK9) (Swiss-Model at <http://expasy.org>) shows a different orientation of the loop that could propagate along the  $\beta 3$  and  $\beta 4$  sheets and thus alter the interaction of the monomers in the hexameric form of Hfq (data not shown) and affect the stability of the Cd-Hfq hexamer.

## DISCUSSION

Expression of Hfq is tightly regulated at both transcriptional and post-transcriptional levels (Tsui and Winkler 1994; Vecerek et al. 2005; Ziolkowska et al. 2006), and its availability is a limiting factor in sRNA-mediated silencing and activation of gene expression (Wagner 2013). To avoid any artifact due to overproduction, sudden induction of Hfq synthesis or formation of heterohexamers, we chose to express the Ec- and Cd-*hfq* genes in the *hfq* mutant at physiological levels under the control of the native *E. coli hfq* promoters and translation initiation sites. This is in contrast to previous studies where Hfq was synthesized from inducible promoters (Vecerek et al. 2008; Boggild et al. 2009; Salim et al. 2012).

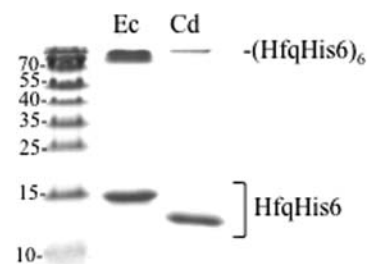
We demonstrate that the Hfq protein of *C. difficile* can substitute for *E. coli* Hfq in the positive control of RpoS, the negative control of OppA expression, and in *hfq* autoregulation showing that it does act as an RNA chaperone, at least in *E. coli* for these functions. However, it is not functionally identical to Ec-Hfq, since Cd-Hfq is unable to carry out the negative control of *ptsG* expression. A possible explanation for this difference is discussed below.

Mutagenesis analysis has identified different conserved amino acids of Hfq implicated in regulation of mRNAs by sRNAs. These amino acids have defined three regions in

the Hfq structure, two important for the interaction with sRNAs, the proximal face and lateral surface, while the proximal face is thought to contact the mRNA (Mikulecky et al. 2004; Ziolkowska et al. 2006; Sauer et al. 2012; Zhang et al. 2013). As described above, the Hfq protein of *C. difficile* retains most but not all the critical residues found to be essential for the function of the *E. coli* protein. Furthermore, the C-terminal domains are very different. By comparing both the full size and C-terminal deleted versions of Ec-Hfq and Cd-Hfq, we have also investigated whether the C-terminal region has a role in Hfq's RNA chaperon function.

We examined the role of Hfq in various sRNA-dependent gene regulations in *E. coli*, which implicate different binding surfaces of the protein. ArcZ regulation of *rpoS* expression requires Q8, F39, F42, K56, H57 on the proximal face, Y25 and I30 on the distal face, and R16 at the rim (Zhang et al. 2013). Among these essential residues only F42 is missing in Cd-Hfq but replaced by Y, another aromatic amino acid. We found that RpoS expression is fully activated in Cd-Hfq containing strains, indicating that Cd-Hfq actively cooperates with the sRNAs in charge of regulating RpoS expression. Our previous results indicated that different sites of Hfq were involved in the control of RpoS and OppA, since mutation of Ec-Hfq Valine 43 to Arginine abolished regulation of RpoS expression but did not impair OppA control (Ziolkowska et al. 2006). In Cd-Hfq V43 is replaced by an Isoleucine and in the case of Ec-Hfq a Cystein at this position is fully compatible with RpoS repression (Ziolkowska et al. 2006).

While Cd-Hfq is as efficient as Ec-Hfq in *oppA* repression and *rpoS* activation of gene expression, it has a greater impact on GcvB, DsrA, and ArcZ sRNAs stability. These three sRNAs are more abundant in Cd-Hfq than in Ec-Hfq containing cells. GcvB coimmunoprecipitates with Hfq and is unstable in its absence, but less than other sRNAs (Zhang et al. 2003, 2013; Pulvermacher et al. 2009; Busi et al. 2010). We show here that GcvB sRNA accumulates to higher levels in the Cd-Hfq containing cells than in the presence of Ec-Hfq. This indicates that Cd-Hfq has a higher protective effect against GcvB degradation than Ec-Hfq. ArcZ is one out of four sRNAs that contributes to *rpoS* translation (Mandin and Gottesman 2010). This sRNA is processed, probably



**FIGURE 6.** The Cd-Hfq hexamer is less stable than the EC-Hfq hexamer. Coomassie staining of an SDS-polyacrylamide gel showing Ec-Hfq and Cd-Hfq proteins after purification.

by RNase E (Papenfert et al. 2009), to ArcZ\*, which is the active form of the sRNA. Surprisingly, full-length ArcZ which is normally undetected in *hfq+* cells, accumulated to high levels in the presence of Cd-Hfq and the processed form ArcZ\*, and was also more abundant especially with the full-size Cd-Hfq. Thus, GcvB, ArcZ, and DsrA are inefficiently processed in the presence of Cd-Hfq. GcvB, DsrA, and ArcZ\* are probably degraded by RNase E when associated with Ec-Hfq as a consequence of their mRNA base-pairing activity, as previously described (Massé et al. 2003; Morita et al. 2005). As RNase E does not exist in *C. difficile*, it is conceivable that Cd-Hfq does not allow recruitment of RNase E in *E. coli*, thus explaining the enhanced stability of certain mRNAs and small RNAs (Monot et al. 2011). In agreement, inactivation of thermosensitive RNase E that stabilizes ArcZ and DsrA in the presence of Ec-Hfq has no impact on their abundance when Cd-Hfq is substituted for Ec-Hfq (Fig. 4C), suggesting that Cd-Hfq may not be proficient in RNase E binding. In the case of *oppA* where the mRNA is as unstable with Cd-Hfq as with Ec-Hfq, degradation of the mRNA would be independent of the formation of the Hfq-sRNA-RNase ribonucleoprotein complex and most probably result from translational inhibition and accessibility of the messenger to the endoribonucleases. Alternatively, both GcvB and ArcZ may interact more tightly with Cd-Hfq than with Ec-Hfq, conferring a higher protection against ribonucleases.

### A role for the C-terminal domain of Hfq

We show that both Ec- and Cd-Hfq proteins are able to negatively control *oppA* expression in the absence of the non-structured C-terminal part of the proteins. This indicates that the two core proteins are stable and functional enough in vivo to regulate the expression of certain targets. Similarly Ec-Hfq and Cd-Hfq core are equally efficient in the control of *ptsG* expression. On the other hand, for other targets the C-terminal region seems to play a role with different graduations: the C-terminal domain of both Ec and Cd Hfq increases the activation of RpoS expression and is required for the autoregulation of Hfq. Those differences could be due to different affinities with the mRNAs. Despite the fact that the C-terminal regions are of different lengths and sequences, they confer similar functions that are not exhibited by the core regions alone. It could be the intrinsically disordered character of this part of the proteins that is required for the interaction with the *rpoS* messenger for both Hfqs.

### The special case for SgrS

Two mechanisms have been proposed to account for sRNA-induced mRNA decay. In the first pathway, interruption of translation frees the messenger of ribosomes, allowing RNase E to access and cleave the mRNA (Morita et al. 2006). In the second pathway, proposed for degradation of *ptsG* and

*sodB* mRNAs, formation of a sRNA-Hfq-RNase E ribonucleoprotein complex stimulates RNase E cleavage of the target mRNA (Morita et al. 2005) even at distance from the interaction site (Prevost et al. 2011). SgrS interaction with *ptsG* mRNA inhibits its translation promoting the rapid degradation of the SgrS-*ptsG* complex (Ikeda et al. 2011). The rapid turnover of *ptsG* mRNA requires the C-terminal scaffold region of RNase E, which interacts with Hfq as well as with Hfq bound to small RNAs (Morita et al. 2005). In addition, RNase E and Hfq bind similar sites on the RNA (Folichon et al. 2003) and base pairing of the sRNA and mRNA may allow Hfq displacement from the sRNA and access of RNase E to the duplex (Massé et al. 2003). We show here that Ec-Hfq carried out repression and degradation of *ptsG* transcript with no role of the C-terminal domain, indicating that the latter is not required for interacting with RNase E. Inhibition of *ptsG* translation and its degradation are not observed in Cd-Hfq-containing cells because in this case SgrS sRNA is not stabilized by Cd-Hfq. This situation is unique compared with the two other sRNAs we have examined here, ArcZ and GcvB, and also GlmZ (data not shown), because all of them are more stabilized by Cd-Hfq than by Ec-Hfq. SgrS sRNA was the first dual-function Hfq-dependent, sRNA regulator identified. It encodes a peptide, SgrT (Wadler and Vanderpool 2007), which is capable of inhibiting  $\alpha$ MG uptake through a mechanism that is independent of the base-pairing function. If translation of SgrT is efficient in the presence of Cd-Hfq even when *ptsG* mRNA is not degraded, this may explain why Cd-Hfq-containing cells can survive in the presence of  $\alpha$ -MG (Fig. 5A).

### Similarities and differences of Ec-Hfq and Cd-Hfq

As discussed above, Ec-Hfq residues are important for ArcZ function, Q8, F39 (proximal face), Y25, and I30 (distal face) (Fig. 1; Zhang et al. 2013) are also present in Cd-Hfq, except that I30 is substituted by a Valine, which does not impact on ArcZ function in *E. coli*.

The proximal face of Hfq (Q8, D9, F39, F42, Y55, K56, H57) is important for SgrS function. It binds the terminator poly(U) tail. This interaction is crucial for PtsG silencing since a SgrS variant with a short poly(U) tail was inactive (Otaka et al. 2011). Q41 and F42 participate in the direct recognition of uracil (Sauer and Weichenrieder 2011). They are replaced by S41 and Y42 in Cd-Hfq. However, replacement of Q41 by S41 in Ec-Hfq neither stabilizes *ptsG* transcript nor destabilizes SgrS (Fig. 5). So, loss of one of the two U-contacting amino acids is not sufficient to prevent SgrS stabilization.

It has been recently demonstrated that conserved arginines on the rim of *E. coli* Hfq protein (RRER motif) are required for its chaperon activity. An interesting correlation has been emphasized between the number of positively charged amino acids (arginines or lysines) at the rim and the function of Hfq from Gram-positive bacteria in sRNA



regulation (Panja et al. 2013). Indeed, among the Hfq proteins studied in Gram-positive bacteria, *L. monocytogenes* Hfq carries the RKEK rim motif and stabilizes at least one sRNA–mRNA complex (Nielsen et al. 2010, 2011), while *B. subtilis* Hfq having a RKEN motif associated with numerous RNAs was not required for sRNA regulation (Heidrich et al. 2007; Gaballa et al. 2008; Dambach et al. 2013). The function of *S. aureus* Hfq carrying the KANQ rim motif remains unclear (Bohn et al. 2007). Interestingly, the Cd-Hfq carries the most conserved RKER motif with just one amino acid substitution as compared with Ec-Hfq (Fig. 1), and thus maintains a similar surface charge with two arginines on the rim as the *E. coli* protein. It is interesting to correlate this conserved positive charge with the similar RNA chaperone functions of this Hfq homolog that we have demonstrated in this work.

On the other hand, there are some differences between the two Hfq proteins that may account for the reduced stability of the hexameric Cd-Hfq chaperone detected in our preparations. First, the presence of an extra amino acid at the junction of the Sm1 and Sm2 motifs in Cd-Hfq as compared with Ec-Hfq may modify the interactions of monomers in the hexameric form (Fig. 1). Second, the sequence of the C-terminal domain may also have an impact on the hexamer organization in agreement with our previous data, suggesting that in Ec-Hfq residues 84–102 protect the interface between monomers and possibly contribute to the thermodynamic stabilization of the hexameric Hfq structure (Arluison et al. 2004). Comparative studies of in vitro properties of these two proteins will be useful to compare the structure and the function of these RNA chaperons.

## Perspectives

While the function of Hfq in Gram-negative bacteria is now well-documented, its role in Gram-positive bacteria is still under debate. Hfq was shown to act as a virulence factor in several bacterial pathogens but it is not at all clear how it acts or whether it cooperates with sRNAs to control physiological functions. In Gram-positive bacteria containing Hfq such as *B. subtilis*, *S. aureus*, and *L. monocytogenes*, the vast majority of the sRNA–mRNA duplexes characterized in vivo do not require Hfq (for review, see Brennan and Link 2007; Toledo-Arana et al. 2007; Repoila and Darfeuille 2009; Hajnsdorf and Boni 2012)). Here we show that Cd-Hfq is functional in sRNA-mediated regulation in *E. coli*. However, its role in *C. difficile* remains to be determined. Numerous potential *trans*-riboregulators have been identified in *C. difficile* by global deep sequencing (Soutourina et al. 2013). Indeed, among the 250 potential regulatory RNAs detected experimentally (with expression of 35 sRNAs confirmed by gene-specific experimental approaches), about 100 sRNAs are located in intergenic regions and might represent *trans*-RNA regulators. These identified RNAs could require Hfq for their regulatory action as observed in many other bacterial

systems (Vogel and Luisi 2011). The growth phase-dependent expression of some of the identified sRNAs and the conservation of the majority of them within *C. difficile* strains strongly suggest that they have a functional role and could impact the physiopathology of this pathogen. Together with the present work showing that Cd-Hfq protein can function as an RNA chaperone, these data emphasize the potential importance of RNA-based regulatory mechanisms in *C. difficile*. Further studies will be required to assess the function of these new sRNA regulators as well as to identify their targets, mechanisms of action, and to determine the role of Hfq protein in related regulatory processes.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Supplemental Table S1 and the primers in Supplemental Table S2. New strains were constructed by P1 transduction.

The plasmids pCd-Hfq, pCd-Hfq core, pEc-Hfq core, and pEc-HfqQ41S used to complement the deletion of the *hfq* gene in *E. coli* were constructed by a two-step PCR procedure from the original pTX381 plasmid containing the *miaA'*-*hfq*-*hflX'* region (Tsui et al. 1994). The first step was made of individual PCRs. The second step combined the former individual PCRs. The product of this PCR is then cleaved by restriction enzymes and recloned in the same sites of pACYC184.

### - pCd-Hfq

The first step is made of three individual PCRs. The first PCR amplifies the 5' UTR of the *E. coli hfq* gene (primers JC411 out and JC400 with pTX381 as the template). The second PCR allows the amplification of the Cd-Hfq ORF from the ATG to the stop codon with primers JC401 and JC414 and *C. difficile* chromosomal DNA as the matrix. The third PCR amplifies a sequence corresponding to 30 nt of the 3' part of Cd Hfq ORF together with sequence of the plasmid pCA24N (Kitagawa et al. 2005) with primers JC413 and JC412out and pCA24N as the template. The second step is a PCR combining the three former PCRs with primers JC411out and JC412out.

### - pCd-Hfq core construction

The first step is made of two individual PCRs. The first PCR is performed using primers JC411out and JC416 with pCd-Hfq as the template, which allows the amplification of the Cd *hfq* gene to the position corresponding to G68 codon with two stop codons. The second PCR is performed with primers JC415 and JC412out and pCd-Hfq DNA as the matrix. The second step combined these two PCR products with primers JC411out and JC412out.

### - pEc-Hfq core

The first step is made of two individual PCRs. The first PCR is performed with primers JC411out and JC418 with pTX381 as the

template. The second PCR is performed with primers JC417 and JC412out and pCA24N DNA as the matrix. We then used these two PCR products with primers JC411out and JC412out.

For these three constructions the PCR products were then cleaved by BamHI and cloned into the corresponding site of pACYC184 plasmid.

### - pEc-HfqQ41S

The first two PCRs allow the amplification of Ec-Hfq in two parts with the pairs of oligos JC425–JC427 and JC426–JC428 and pTX381 as template, with JC425 and 426 harboring the Q41S mutation. The two PCR products were used with primers JC427 and JC428 in a second set of amplification. The resulting product was cleaved with HindIII and XmaI and cloned into the corresponding sites of pTX381.

### - pCd-HfqHis6

The *C. difficile* *hfq* gene was amplified by PCR from *C. difficile* strain 630 DNA using oligonucleotides JC407 and JC408. The 280-bp amplified product was cloned into the PCRII blunt vector (Invitrogen), giving pTOPO-Cd-HfqHis6. The 263-bp NcoI–XhoI fragment of pTOPO-Cd-HfqHis6 was then cloned between corresponding sites of the pET28a expression vector downstream from the T7 promoter. The resulting plasmid pET28a-Cd-HfqHis6 allows expression in *E. coli* BL21  $\lambda$ DE3 $\Delta$ *hfq* of the *C. difficile* *hfq* protein bearing a C-terminal His-tag (Cd-HfqHis6).

### $\beta$ -Galactosidase assay

$\Delta$ *hfq* derivative of RO91 containing the *rpoS::lacZ* chromosomal fusion (Lange and Hengge-Aronis 1994) and  $\Delta$ *hfq* derivative of IBhfq95 containing the *hfq::lacZ* chromosomal fusion (Supplemental Table S1) were transformed with the pACYC184 series of *hfq*-expressing plasmids described above. Cells were grown at 37°C in Luria-Bertani (LB) medium with chloramphenicol. Cells were harvested in exponential phase, disrupted by sonication, and  $\beta$ -galactosidase activities were measured in clarified cell extracts. Specific  $\beta$ -galactosidase activity was expressed as nmol ONPG (o-nitrophenyl- $\beta$ -D-galactopyranoside) hydrolyzed/min/mg of total soluble cell proteins (Ziolkowska et al. 2006). Protein concentrations in lysates were measured by Bradford assay (Bio-Rad). Means values are calculated from three independent experiments.

### Protein purification and band-shift assay

Ec-HfqHis6 and Cd-HfqHis6 were overexpressed and purified in parallel as previously described (Ziolkowska et al. 2006). Templates for the synthesis of the different sRNAs were obtained by PCR amplification using the primers described in Supplemental Table S2. Band-shift assays were performed as in Folichon et al. (2005) with Hfq concentration expressed on the basis of the hexamer form. The radioactivity signal above the level of the free RNA was counted as retarded RNA on the gel. Data were analyzed and fitted to the Hill equation using Kaleidagraph and the  $K_{1/2}$  (half saturation values) were derived from the fit of the data.

### RNA preparation, analysis, and labeling

Total RNA was prepared from bacteria grown to an  $A_{650} = 0.35$ – $0.4$  in LB medium using the hot-phenol procedure described in Braun et al. (1996). Ten micrograms of total RNA were separated either on 1% agarose formaldehyde gel or 6% polyacrylamide gels for mRNA and sRNA analysis, respectively, and analyzed by Northern blotting (Hajnsdorf et al. 1994; Hajnsdorf and Régnier 1999). Templates for the synthesis of ArcZ, GcvB, SgrS, and *oppA* and *ptsG* RNA probes were obtained by PCR amplification using an oligonucleotide containing the T7 promoter and a RNA-specific oligonucleotide (indicated with prefix m) described in Supplemental Table S2. RNAs were synthesized by T7 RNA polymerase with [ $\alpha$ - $^{32}$ P]UTP yielding uniformly labeled RNAs (Hajnsdorf and Régnier 2000). Membranes were also probed for 5S rRNA with a 5'-labeled oligonucleotide (Supplemental Table S2). RNA levels were quantified by a PhosphorImager. *hfq* RNA levels were determined by abortive reverse transcription using 5'-labeled hfg primer (Supplemental Table S2) as described in Hajnsdorf et al. (1995) except that reverse transcription was carried out with 0.25 mM dATP, 0.25 mM dGTP, 0.25 mM dTTP, and 0.25 mM ddCTP. RNA levels were quantified using a PhosphorImager.

### SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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