The EMBO Journal (2004) 23, 396–405 | © 2004 European Molecular Biology Organization | All Rights Reserved 0261-4189/04
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THE
EMBO
JOURNAL

Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator

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The Sm-like protein Hfq is involved in post-transcriptional regulation by small, noncoding RNAs in *Escherichia coli* that act by base pairing. Hfq stabilises the small RNAs and mediates their interaction with the target mRNA by an as yet unknown mechanism. We show here a novel chaperoning use of Hfq in the regulation by small RNAs. We analysed *in vitro* and *in vivo* the role of Hfq in the interaction between the small RNA RyhB and its *sodB* (iron superoxide dismutase) mRNA target. Hfq bound strongly to *sodB* mRNA and altered the structure of the mRNA, partially opening a loop. This gives access to a sequence complementary to RyhB and encompassing the translation initiation codon. RyhB binding blocked the translation initiation codon of *sodB* and triggered the degradation of both RyhB and *sodB* mRNA. Thus, Hfq is a critical chaperone *in vivo* and *in vitro*, changing the folding of the target mRNA to make it subject to the small RNA regulator.

The EMBO Journal (2004) **23,** 396–405. doi:10.1038/sj.emboj.7600058; Published online 22 January 2004
Subject Categories: RNA; proteins
Keywords: iron superoxide dismutase; protein–RNA interaction; regulatory RNA; RNA chaperone; RNA–RNA interaction

Introduction

Hfq is a small, abundant, ubiquitous protein. It was originally described in the early 1970s as a host factor necessary for replication of the RNA phage Qβ (Franze de Fernandez *et al*, 1968), but has now been shown to be a pleiotropic regulator of the expression of many genes by binding to RNAs (Muffler *et al*, 1997; Tsui *et al*, 1997). Hfq affects the stability of several mRNAs (Tsui *et al*, 1997), and targets them for degradation by increasing polyadenylation (Hajnsdorf and Régnier, 2000; Le Derout *et al*, 2003), interfering with ribosome binding and with translation (Vytvytska *et al*, 2000). It appears to bind preferentially to unstructured A/U-rich sequences, frequently close to more structured regions of the RNA (Møller *et al*, 2002; Zhang *et al*, 2002), and is similar to eukaryotic Sm-like proteins in both sequence and structure (Møller *et al*, 2002; Zhang *et al*, 2002). Hfq also interacts with several small RNAs (DsrA, RyhB, Spot42 RNA, OxyS) by base pairing and is required for their function (Sledjeski *et al*, 2001; Massé and Gottesman, 2002; Møller *et al*, 2002; Zhang *et al*, 2002). Hfq may also, by targeting mRNAs and sRNAs, act as an RNA chaperone (Zhang *et al*, 2002; Moll *et al*, 2003), but how it does so is not well defined.

Massé and Gottesman (2002) discovered the RNA RyhB in a genome-wide search for small regulatory RNAs. They showed that RyhB downregulated the synthesis of a set of iron proteins, including iron superoxide dismutase (FeSOD), and that its production is controlled by the Fur protein (ferric uptake repressor). Fur represses the transcription of numerous genes involved in (or related to) iron metabolism. Fur acts with ferrous iron as cofactor and is inactive in iron starvation conditions, leading to the expression of controlled genes. However, some genes, including *sodB*, which encodes FeSOD, are underexpressed in *fur* mutants, but why this is so was not elucidated. Studies on *sodB* regulation (Dubrac and Touati, 2000, 2002) showed that *sodB* mRNA is much less stable in *fur* mutants, suggesting that Fur-mediated 'positive regulation' is indirect and acts post-transcriptionally; however, the mechanism remains elusive. The finding of RyhB solves this question.

How RyhB first interacts with its targets has not been determined. Some regions of RyhB are complementary to regions on its mRNA targets. They lie around the translation initiation site, suggesting a pairing that inhibits translation. The degradation of both RyhB and its target is RNase E-dependent. RyhB is degraded as being used, most likely as a consequence of pairing with its target. RyhB is extremely unstable in the absence of Hfq (Massé *et al*, 2003). *In vivo*, Hfq is required for RyhB activity: an *hfq* mutation has been found to alleviate the repression of *sodB* in the *fur* mutant (D Touati, unpublished). But despite the advance made in elucidating the Fur-RyhB regulation of *sodB*, we still know very little about the initial interaction between RyhB and its *sodB* mRNA target, or about the way in which Hfq interferes with this interaction.

This structural analysis was carried out to delineate the binding sites on the *sodB* and RyhB RNAs for Hfq, and the domains involved in the interaction between the two RNAs. We investigated the effect of Hfq binding to its targets. We find that Hfq binds strongly to *sodB* mRNA, leading to a change in the structure of the RNA that renders it accessible to RyhB. In contrast, when Hfq binds to RyhB, it has no effect on the secondary structure of the RNA. We also studied the effects of Hfq on the expression of a *sodB-lacZ* fusion product from wild-type and mutated *sodB* mRNA. Our results indicated that Hfq plays a critical role, acting as a chaperone to *sodB* mRNA.

Results

Strong binding of Hfq to sodB mRNA and weak binding to RyhB RNA

Although Hfq is required for the interaction of many small RNAs with their RNA targets, the mechanism involved is

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Received: 29 September 2003; accepted: 8 December 2003; Published online: 22 January 2004

unclear and may depend on the target. We therefore investigated the role of this protein in *sodB* regulation. We analysed the specific binding of Hfq to RyhB and *sodB* mRNA in gel mobility shift assays (Figure 1). Hfq interacted very strongly with *sodB* mRNA, with a dissociation constant K_D of 1.8 nM. But Hfq was bound much less strongly to RyhB, with a K_D of about 1.5 μM, which is of the same order of magnitude as that for the binding of Hfq to DsrA, OxyS or Spot 42 RNA (Sledjeski *et al*, 2001; Møller *et al*, 2002; Zhang *et al*, 2002; Brescia *et al*, 2003). Thus, binding experiments suggest that Hfq is necessary for regulation of gene expression by small RNAs, at least for *sodB*, not just by stabilising the small RNA, but via a strong interaction with the mRNA target. We therefore further investigated the effects of Hfq binding to *sodB* mRNA, to determine whether and how this binding interfered with regulation by RyhB.

Identification of the Hfq-binding site on sodB mRNA

An A/T-rich region just upstream from the Shine–Dalgarno sequence of *sodB* plays an important role in *sodB* regulation (Dubrac and Touati, 2000). The A/U-rich RNA sequence is a single-stranded linker between two stem-loops. The first stem-loop (stem-loop **a**) begins with the first transcribed nucleotide. Partial deletion of this structure stabilises the mRNA but does not abolish Fur (RyhB) regulation (Dubrac and Touati, 2000). The second stem-loop (stem-loop **b**) encompasses the region in which translation is initiated: the Shine–Dalgarno sequence is part of the stem and the AUG start codon lies in an internal loop (Figure 2A). We postulated that the linker between the two stem-loop structures could be an Hfq-binding site, as Hfq binds preferentially to A/U-rich stretches in RNA (Møller *et al*, 2002; Zhang *et al*, 2002).

We therefore constructed *sodB* RNA fragments with deletions of the 5′ region, and used these fragments to compete against labelled $sodB_{1-148}$ fragments in Hfq binding assays (Figure 2). Competition occurred only if the A/U-rich region was present (fragments $sodB_{1-148}$, $sodB_{20-148}$, $sodB_{31-148}$) and not if this region was deleted (fragments $sodB_{45-148}$, $sodB_{56-148}$) or partially replaced by G/C nucleotides (fragment $sodB_{AT}$). We also constructed fragments with different 3′ end run-off zones (fragments $sodB_{1-50}$, $sodB_{1-81}$). Fragment $sodB_{1-50}$, which has the complete A/U-rich region but lacks part of stem-loop **b**, did not compete against labelled $sodB_{1-148}$.

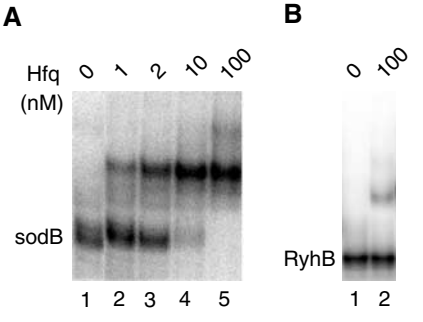

Figure 1 Hfq binding to *sodB* and RyhB RNA. (**A**, **B**) In all, 0.1 nM of [α-^{32}P]UTP-labelled $sodB_{1-148}$ (A) or RyhB (B) transcript was incubated without or with various concentrations of purified Hfq (indicated above the gel) in the presence of 100 ng/μl tRNA. After incubation for 5 min at 37°C, the mixture was analysed by electrophoresis in a native polyacrylamide gel.

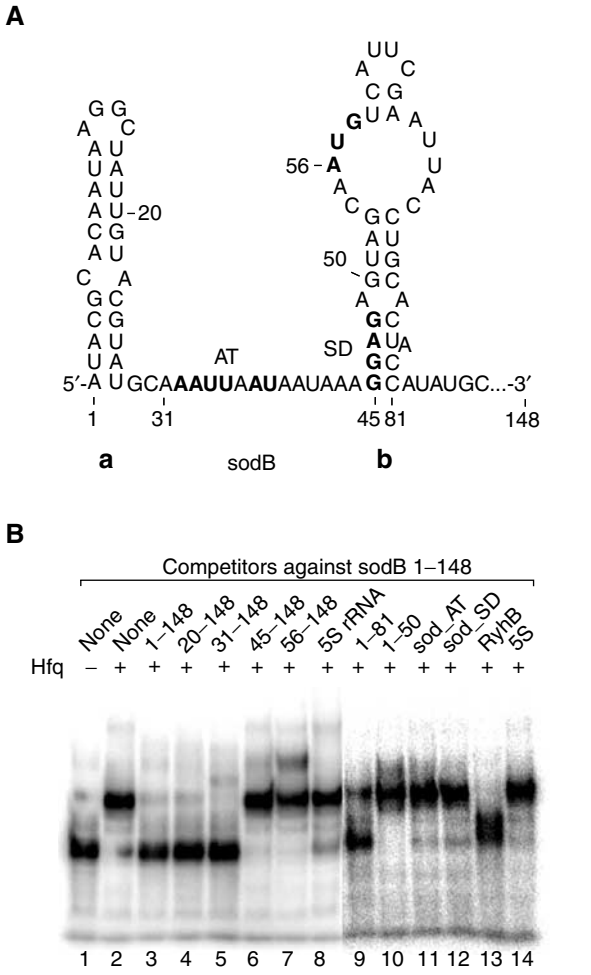

Figure 2 Binding of *sodB* mRNA and competitors to Hfq. (**A**) Secondary structure of the 5′ end of *sodB* mRNA, as predicted by the *mfold* program. Transcription begins at nucleotide 1 and translation is initiated at nucleotide 56. The AUG start codon is shown in bold. The numbering indicates the positions of the truncated *sodB* RNA fragments used as competitors in gel mobility shift assays. The nucleotides exchanged during construction of the mutations in the A/T-rich region (AT) or of the Shine–Dalgarno sequence (s.d.) are shown in bold. The stem-loops of *sodB* are indicated by lowercase letters in bold typeface. (**B**) Gel mobility shift assay with truncated *sodB* mRNA fragments competing against $sodB_{1-148}$ RNA for Hfq binding. Labelled $sodB_{1-148}$ transcript was incubated with (+) or without (−) Hfq protein. Unlabelled competitor RNA was added at 5000-fold molar excess. The competitor added in each case is indicated at the top of the lane.

Similarly, a fragment ($sodB_{SD}$) in which mutations altered the secondary structure of the stem was not competitive. Thus, Hfq binds to the A/U-rich region, but stem-loop **b** is required for binding.

We performed a minimal binding analysis to confirm these findings. The 5′ or 3′ end-labelled $sodB_{1-148}$ RNA was subjected to partial alkaline hydrolysis, and the binding of the fragments to Hfq was analysed (Figure 3). 5′ end-labelled fragments up to 80 nucleotides long remained unbound, whereas longer RNAs were bound to Hfq. Position +81 corresponds to the last nucleotide of stem-loop **b**. 3′ end-labelled fragments were bound if the A/U-rich region was present. This again indicates that the A/U-rich region is necessary but not sufficient for binding; Hfq binding requires

the presence of stem-loop **b**. This is consistent with the results obtained in the gel mobility shift assays. Stem-loop **a** was neither necessary nor sufficient for Hfq binding.

The binding of Hfq to RyhB stabilises it *in vivo* (Massé *et al*, 2003). RyhB is 90 nucleotides long, and has a stretch of five A/U residues at position 61–65 between two stem-loops (Figure 3D). RyhB 5′ end-labelled fragments longer than 65 nucleotides and 3′ end-labelled fragments of at least 30 nucleotides were bound in the minimal binding assay (Figure 3B). These results suggest that Hfq binds to the A/U linker at position 61–65 if either of the stem-loops is present, with no preference for a particular stem-loop. These results are similar to those for the binding of Hfq to OxyS (Zhang *et al*, 2002) or DsrA (Brescia *et al*, 2003).

Hfq alters the secondary structure of sodB mRNA, but not that of RyhB

We further investigated the role of Hfq in the RyhB-mediated regulation of *sodB* by determining whether the binding of Hfq modified the secondary structures of *sodB* mRNA and RyhB. We carried out RNase footprinting with various specific nucleases. RNase A cleaves at a position 3′ to single-stranded C and U residues, whereas RNase T_1 cleaves after unpaired G residues. RNase I cleaves single-stranded RNA without nucleotide specificity. RNase V_1 recognises predominantly double-stranded regions, although stacked single-stranded structures or pseudoknots are also cleaved by this enzyme. Cleavage by nucleases may induce conformational rearrangements leading to secondary cleavages. We therefore carried out all experiments with several concentrations of nucleases and/or incubation times. Results were confirmed by footprinting with 3′-labelled substrates (data not shown).

The secondary structure of *sodB* mRNA deduced from this analysis was consistent with the conformation predicted by the *mfold* program (Zuker, 2003) (Figure 4C). RNase V_1 cleavage of nucleotides 4, 5, 7–10 and 18–21 and 24–27, and the T_1 cleavage of residues 14 and 15 confirmed the identification of stem-loop **a** (Figure 4A). RNase I cleavage was detected for the A/U-rich region (in the absence of Hfq), followed by a stretch of 9 nucleotides that was not cleaved. The next 18 nucleotides were again cleaved (more readily in the presence of Hfq, especially nucleotides 59–60) by RNase

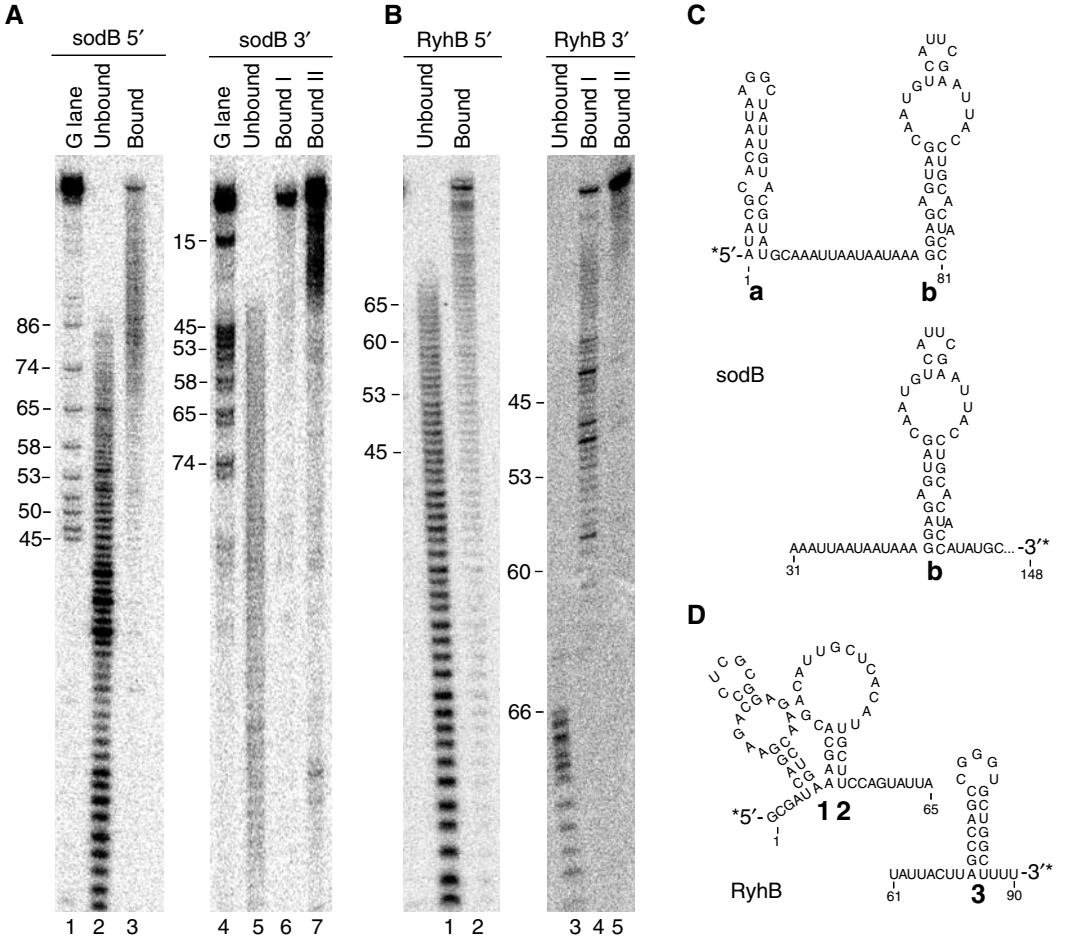

Figure 3 Minimal requirements for *sodB* and RyhB RNA binding to Hfq. (**A**, **B**) Minimal binding assay. 5′ or 3′ end-labelled $sodB_{1-148}$ RNA (A) and RyhB RNA (B) was subjected to partial alkaline hydrolysis. The resulting fragments were then incubated with Hfq, and bound and unbound fragments were separated by native polyacrylamide electrophoresis and fractionated on a denaturing polyacrylamide gel. In the experiments with 3′ end-labelled RNAs, two different complexes of bound RNA were detected and fractionated separately. The numbers to the left indicate sequence positions with respect to the transcription start site. (**C**, **D**) Summary of the minimal binding assay. The minimal 5′ and 3′ end-labelled $sodB_{1-148}$ (C) and RyhB (D) RNA sequences that bound to Hfq are shown. The stem-loops of *sodB* are indicated by lowercase letters in bold typeface, and the stem-loops of RyhB are indicated by numbers in bold typeface.

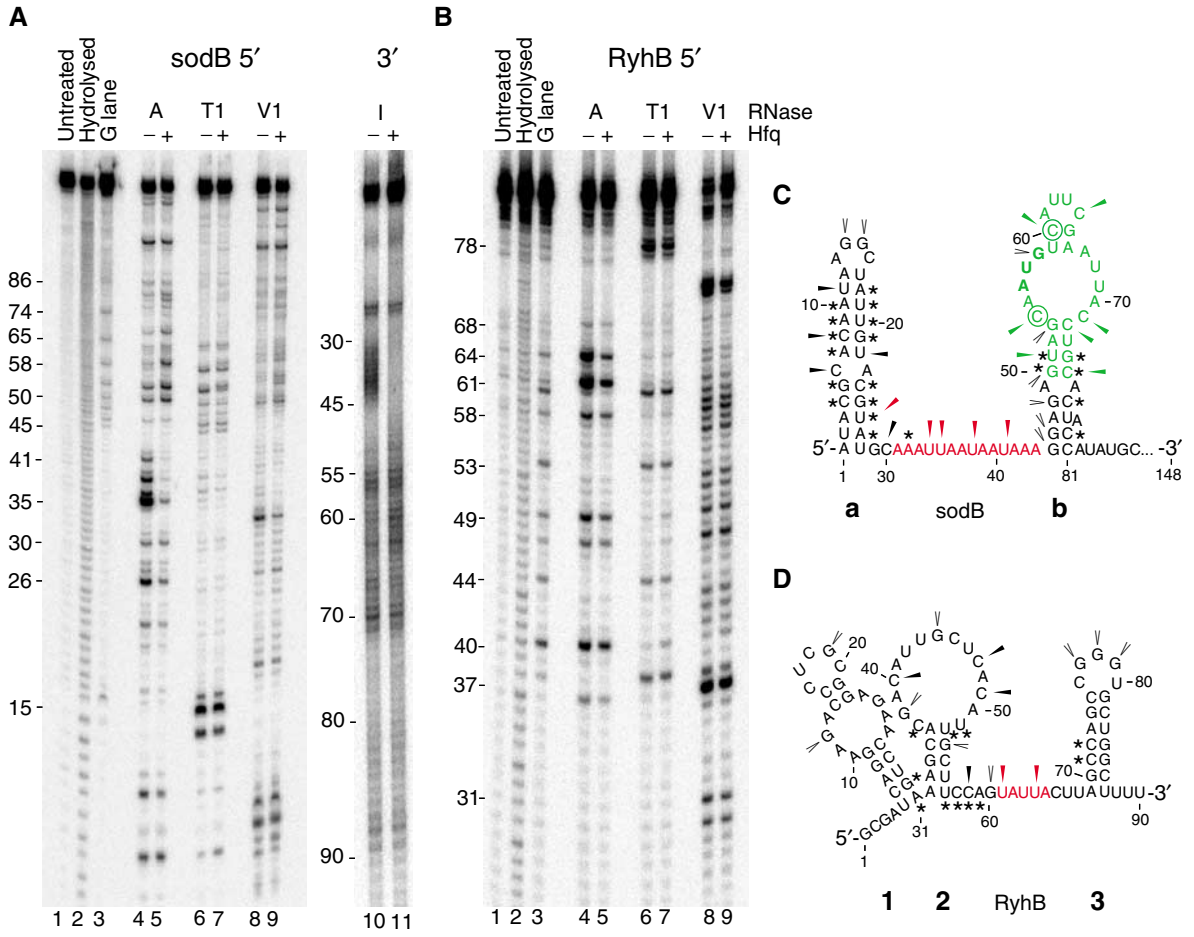

Figure 4 Changes in RNA structure upon Hfq binding. (**A**) RNase footprinting. 5′ end-labelled $sodB_{1-148}$ (A) or RyhB (**B**) transcript was subjected to partial digestion with RNase A, RNase T_1 or RNase V_1, with (+) or without (−) purified Hfq protein. In (A), 3′ end-labelled $sodB_{1-148}$ was digested with RNase I. The resulting fragments were then analysed on a denaturing sequencing gel. The numbers to the left indicate sequence positions with respect to the transcription start site. (**C**, **D**) Summary of the RNase footprints of $sodB_{1-148}$ (C) and RyhB (D) RNA. Elongated triangles indicate RNase A cleavage sites. Arrowheads indicate RNase T_1 cleavage sites, and asterisks indicate RNase V_1 cleavage sites. Nucleotides for which cleavage was more or less likely to occur in the presence of Hfq are shown in green and red, respectively. Circled residues indicate unusual cleavage sites for RNase T_1 upon Hfq binding. Secondary structures were predicted with the *mfold* program, based on the results of footprinting experiments.

I, while afterwards a stretch of noncleavage followed. The cleaved nucleotides correspond to loop **b** between the uncleaved nucleotides corresponding to stem **b**.

The A/U-rich region between nucleotides 31 and 44 displayed fewer cleavages by RNase A in the presence of Hfq and was not cleaved by RNase I, indicating that Hfq bound to and protected this region, consistent with the results obtained in the binding analysis. Adding Hfq changed the cleavage pattern in the stem-loop **b** region. The RNase A cleavage of nucleotides 51, 54 and 60 was enhanced. RNase T_1 cleaved the molecule after nucleotides 54 and 60 (C residues), and RNase V_1 cleaved at nucleotides 55 and 61. RNase I cleavage of the nucleotides 59–60 was enhanced. We interpret these results as indicating melting of the double-stranded nucleotides 50–53/72–75 and 59–60/65–66, opening the internal loop to give a larger terminal loop. Based on the unusual pattern of cleavage by RNase T_1 and the cleavage of single-stranded nucleotides by RNase V_1, we suggest that Hfq not only opens the loop but also causes stacking of nucleotides in this region. This region, between residues 52 and 60, is complementary to the region between nucleotides 38 and 47 of RyhB and may be essential for the *sodB*–RyhB interaction (see below).

Footprinting analysis of RyhB predicted a slightly different secondary structure (Figure 4) from that proposed based on computer calculations (Massé and Gottesman, 2002). We did not find the linker between stem-loops 1 and 2. Consequently, the linker between stem-loops 2 and 3 is 4 nucleotides longer than previously predicted. The second loop was identical to that previously predicted. Hfq slowed the cleavage of the A/U-rich region between nucleotides 61 and 65, indicating that Hfq bound to and protected this region. In contrast to *sodB* mRNA, there was no other change in cleavage pattern on protein binding, indicating that the binding of Hfq does not alter the structure of RyhB.

Interaction between sodB mRNA and RyhB

An interaction between sRNA and mRNA has been demonstrated for OxyS-*fhlA* and Spot42-*galK* by gel mobility shift assay (Møller *et al*, 2002; Zhang *et al*, 2002). We carried out similar experiments in which we added various amounts of unlabelled *sodB* mRNA (0.1, 0.5, 1 μM) to labelled RyhB

(1 nM) in the presence or absence of 0.1 μM Hfq, and then subjected the mixture to polyacrylamide gel electrophoresis. We detected no complexes between RyhB and *sodB* mRNA, in either the presence or absence of Hfq (data not shown).

We checked that this failure to detect efficient binding of RyhB to its *sodB* mRNA target was not due to some experimental defect by carrying out a control experiment in which we assessed the binding of RyhB to *sdhD* (succinate dehydrogenase) mRNA. The expression of *sdhD* is also repressed by RyhB, and the *sdhD* mRNA has a large region complementary to RyhB (Massé and Gottesman, 2002). Experiments to investigate the interaction between RyhB and *sdhD* mRNA showed a clear shift in the mobility of RyhB (1 nM) when *sdhD* mRNA (10 nM) was added, even in the absence of Hfq, indicating that the experimental conditions were suitable for RNA binding (data not shown). We therefore conclude that the RyhB–*sodB* mRNA interaction is not stable enough for detection in gel mobility shift assays, unlike other interactions between small RNAs and their mRNA targets. We therefore attempted to detect it by secondary structure analysis.

The structure of *sodB* mRNA changed when RyhB was added in the presence of Hfq (Figure 5). Nucleotides 51, 54 and 60 were protected against RNase A cleavage, and residues 50, 53 and 58 were protected against RNase T_1 cleavage. This suggests that *sodB* mRNA binds to RyhB in this region, which is consistent with the complementary nature of the region of *sodB* mRNA between nucleotides 52 and 60 and the region 38–47 of RyhB. The region downstream from nucleotide 50 was also modified, as shown by changes in the pattern of RNase V_1 cleavage. There was no interaction in the absence of Hfq (data not shown).

We also analysed the effect of adding $sodB_{1-148}$ RNA to the labelled RyhB in the presence of Hfq. Adding $sodB_{1-148}$ RNA protected nucleotides 37, 40 and 47 against RNase A, and nucleotides 38 and 44 against RNase T_1 cleavage.

Similar protection assays were carried out with native *sodB* mRNA. The protected region was identical to that detected following the addition of the $sodB_{1-148}$ fragment. We detected no additional sites of interaction between RyhB and *sodB* mRNA. The patterns of RNase V_1 cleavages of $sodB_{1-148}$ fragments and native *sodB* mRNA differed. This may be due to differences in the tertiary structures of the two RNAs.

We therefore conclude that RyhB interacts with *sodB* mRNA only in the presence of Hfq, over a stretch of 9 complementary nucleotides, encompassing the AUG initiation codon of the *sodB* mRNA translation.

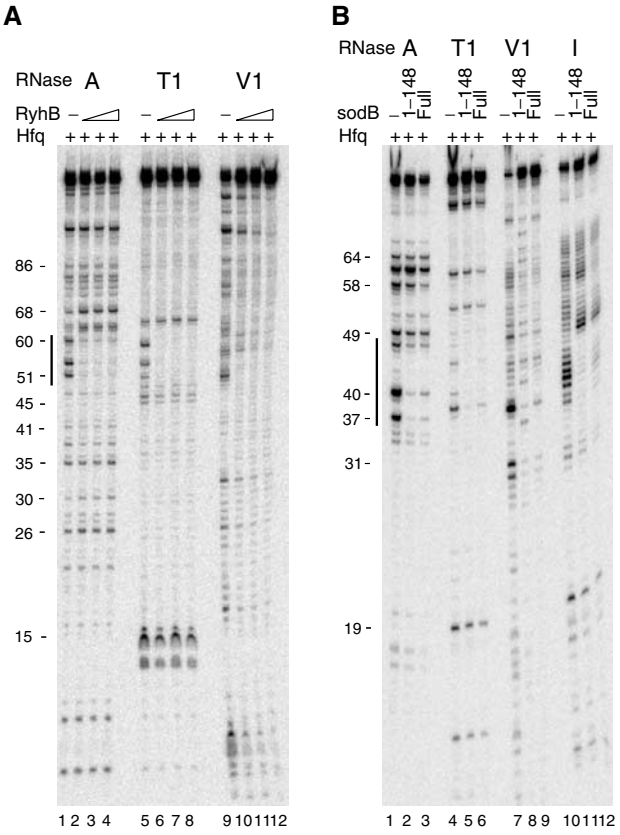

Figure 5 RyhB–*sodB* RNA interaction in the presence of Hfq. (**A**) RNase footprinting. In all, 0.2 pmol of 5′ end-labeled $sodB_{1-148}$ transcript was incubated with Hfq (1 pmol) and then subjected to partial digestion with RNase A, RNase T_1 or RNase V_1, without or with unlabelled RyhB RNA (0, 1, 3 or 5 pmol). The resulting fragments were then analysed on a denaturing sequencing gel. The numbers to the left indicate sequence positions with respect to the transcription start site, and the black vertical line indicates the region protected upon base pairing. (**B**) RNase footprinting. In all, 0.2 pmol of 5′ end-labelled RyhB transcript was incubated with Hfq (1 pmol) and subjected to partial digestion with RNase A, RNase T_1, RNase V_1 or RNase I, with or without unlabelled $sodB_{1-148}$ or full-length *sodB* RNA (5 pmol). The resulting fragments were then analysed on a denaturing sequencing gel. The numbers to the left indicate sequence positions with respect to the transcription start site and the black vertical line indicates the region protected upon base pairing.

Role of Hfq in sodB expression

RyhB expression is under the control of Fur (Massé and Gottesman, 2002), and it is highly expressed in *fur* mutant. In *fur* mutant, *sodB-lacZ* fusion expression is low and the *sodB* mRNA is rapidly degraded (Dubrac and Touati, 2000). The expression and stability of the mRNA are restored to the wild-type level in *fur ryhB* (data not shown) and *fur hfq* (Figure 6). This indicates that Hfq is required for RyhB-mediated repression of *sodB*. This may simply reflect the instability of RyhB in the absence of Hfq (Massé *et al*, 2003). However, our *in vitro* results suggest that the structural modifications induced by the binding of Hfq to *sodB* mRNA are a prerequisite for interaction with RyhB. We therefore investigated whether a defect in the binding of Hfq to *sodB* mRNA could impair repression by RyhB *in vivo*.

We generated constructs encoding fusion proteins, carrying the 1–148 DNA fragment of wild-type *sodB* or the mutated A/T region to which Hfq does not bind in our *in vitro* competition assays. These constructs were named $(sodB\text{-}lacZ)_{19}$ and $(sodB\text{-}lacZ)_{18}$, respectively. Expression of the wild-type fusion, $(sodB\text{-}lacZ)_{19}$, was very poor in the *fur* mutant, and a *ryhB* mutation completely restored expression, as expected (data not shown). A mutation in *hfq* also almost completely restored full expression (Figure 6A). Expression of the fusion $(sodB\text{-}lacZ)_{18}$ with a *sodB* mutated Hfq-binding site was slightly lower than expression of wild-type fusion $(sodB\text{-}lacZ)_{19}$. In the *fur* mutant, expression of $(sodB\text{-}lacZ)_{18}$ was only slightly reduced (Figure 6A). Thus, *in vivo*, the modification of the binding site of Hfq on *sodB* strongly impairs the repression by RyhB. This is consistent with

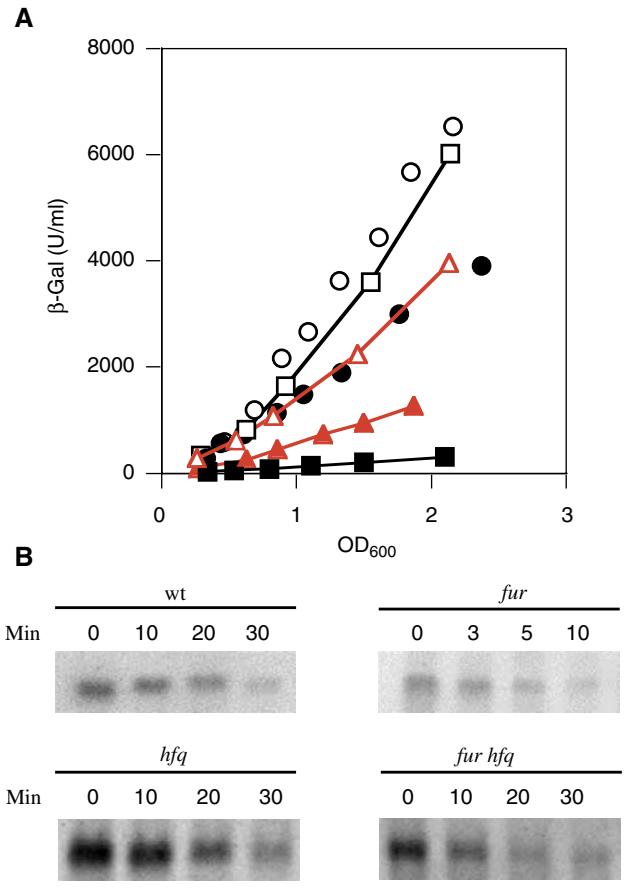

Figure 6 Role of Hfq in *sodB* expression and on *sodB* mRNA stability. (**A**) Strains *fur*$^+$ and *fur*$^-$, carrying the fusion (*sodB-lacZ*)$_{19}$ (black) and the fusion (*sodB-lacZ*)$_{18}$ (red), were grown in LB medium and assayed for β-galactosidase activity. β-Galactosidase activity, expressed in Miller units per millilitre, is plotted against OD_{600} units. The values shown are representative of three experiments for which individual values did not differ by more than 15%. (*sodB-lacZ*)$_{19}$: □, wild type; ■, *fur*; ○, *hfq*; ●, *fur hfq*; (*sodB-lacZ*)$_{18}$: △, wild type; ▲, *fur*. (**B**) Strains containing plasmid pHS1–8 were grown at 37°C to an OD of about 1. Rifampin was added to a final concentration of 150 μg/ml, and samples were taken following incubation at 37°C for Northern analysis. Half-lives estimated from quantitative analysis were: 17.5 min in the wild type and *hfq* strains; 15.5 min in the *fur hfq* strain; and 4.75 min in the *fur* strain.

the important effects observed *in vitro* of Hfq binding on *sodB* mRNA.

Discussion

The protein Hfq is required for regulation by small RNAs that act by base pairing with their mRNA targets. It is thought that binding of Hfq to these small RNAs protects them from degradation and enhances RNA–RNA pairing with their targets. Although chaperoning by Hfq for the interaction of small regulatory RNAs with their mRNA targets is required in several cases, the nature of chaperoning has not been well defined.

We have studied the role of Hfq in the regulation of *sodB* by the small RNA RyhB. We showed a novel use of Hfq in the regulation by small RNA. The binding of Hfq to its mRNA target leads to changes in mRNA structure that are critical for access by the small regulatory RNA.

Role of Hfq binding to its mRNA target

Hfq binds strongly to *sodB* mRNA to a stretch of 14 A/U residues (5′-AAAUUAAUAAUAAA-3′) in the leader sequence of *sodB* mRNA, in the region between two stem-loops. The affinity of Hfq for RyhB RNA is almost three orders of magnitude lower, and the binding site is limited to 5 nucleotides (5′-UAUUA-3′). This affinity is similar to that of Hfq for other small RNAs (Sledjeski *et al*, 2001; Møller *et al*, 2002; Zhang *et al*, 2002; Brescia *et al*, 2003). Hfq specifically requires stem-loop **b** of *sodB* RNA for efficient binding to this molecule. No such specificity for a particular stem-loop has been reported for the small RNAs OxyS (Zhang *et al*, 2002), DsrA (Brescia *et al*, 2003) or RyhB (this study); a stem-loop preceding or following the recognition site is sufficient. The reasons for this specificity are unclear, but it has important consequences. Hfq binding results in the opening of the internal loop of stem **b** to generate a terminal loop, making the messenger accessible to RyhB. The nucleotides in this loop are stacked, as suggested by cleavage by RNases specific for single- and double-stranded RNA at the same site.

Some repression was still observed in the strain with a mutated A/U-rich region. The ratio between expression in the wild type and expression in the *fur* mutant varied from 15.7 for wild-type mRNA to 2.4 for the mutated mRNA. Only part of the Hfq-binding site is modified in this construct and, although Hfq does not bind to the mutated RNA *in vitro* in the competition assay, some residual binding may occur. However, the expression of the *sodB-lacZ* fusion was slightly (1.6-fold) lower in the *hfq fur* mutant than in the *hfq* strain, indicating that RyhB still has some effect on *sodB* even in the absence of Hfq. This minor effect was not detected in our footprint assays. Thus, *in vivo* and *in vitro* data support the idea that the binding of Hfq to *sodB* mRNA renders it accessible to RyhB. We proposed that the binding of Hfq opens stem-loop **b** (model in Figure 7). However, anomalous cleavages in the region of stem-loop **b** suggest that the stem structure is not stable. Stability of the (truncated) stem after the opening of the stem-loop would imply that Hfq itself provides significant stabilisation. This is consistent with the absence of regulation by RyhB in strains with a mutated Hfq-binding site and in *hfq* mutants. Thus, although alternative structures for *sodB* mRNA cannot be excluded, our data are consistent with the model.

We conclude that Hfq acts as an mRNA chaperone in the *sodB*–RyhB interaction, altering the structure of the messenger and presenting it to the repressor.

Interaction between RyhB and sodB RNA

Our gel mobility shift assays showed that RyhB interacts with *sdhD* mRNA, but not with *sodB* mRNA. However, we detected an interaction with *sodB* mRNA in structural analysis by footprinting. This failure to detect an interaction in gel mobility shift assays may be because the interaction is too unstable to be detected in this assay. RyhB did not give a sharp signal in the competition assay against *sodB* (see Figure 2B, lane 13). This may indicate an interaction between the two RNAs, and also that Hfq is no longer bound to them. We have shown that stem-loop **b** of *sodB* mRNA is important for the binding of Hfq. RyhB interacts with this stem-loop, causing structural changes within it that may release Hfq from *sodB* mRNA. Thus, RyhB may interact differently with different targets. Although the mechanism underlying the

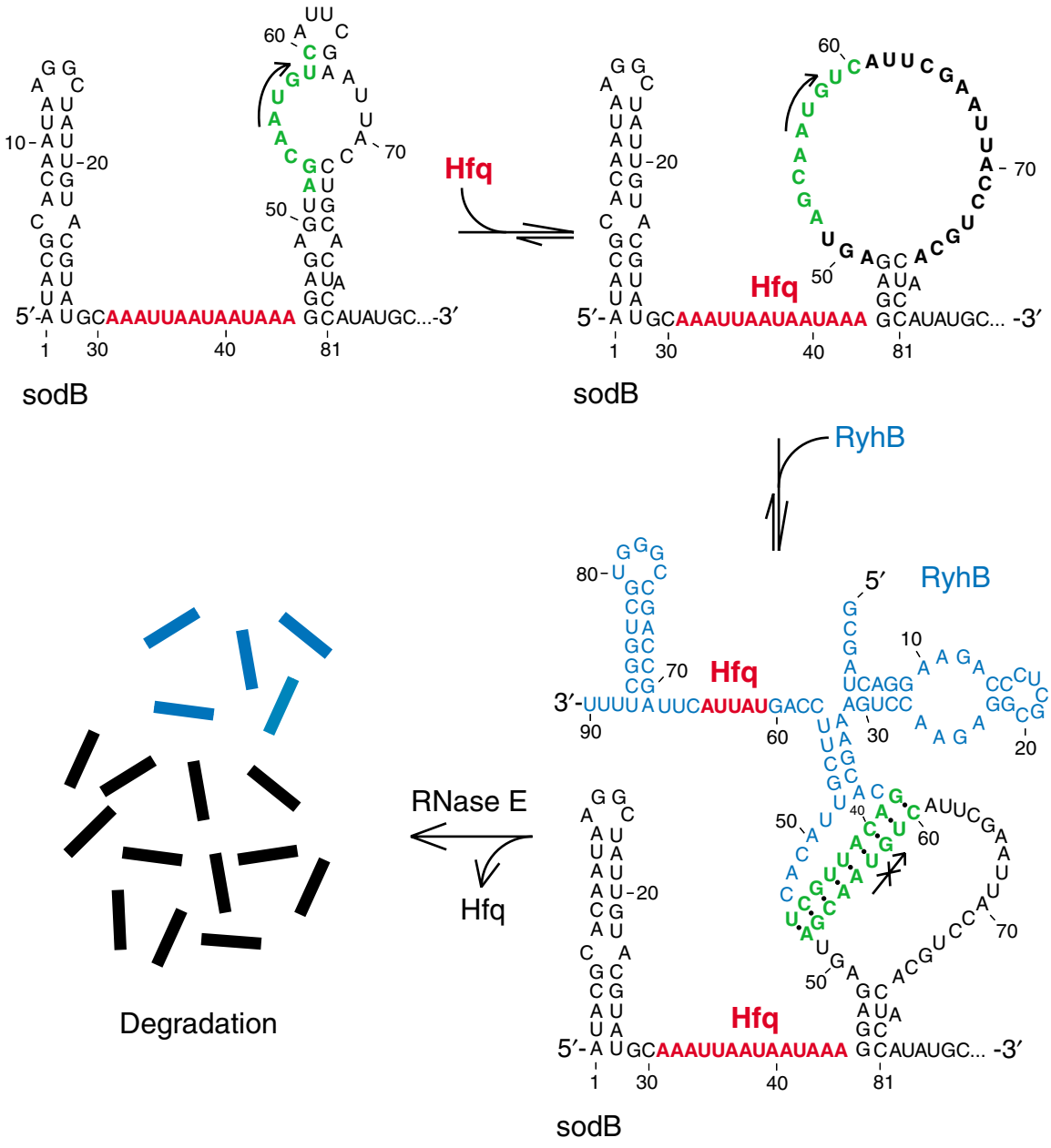

Figure 7 Model for *sodB* mRNA–Hfq–RyhB interaction. Hfq binds with high affinity to *sodB* mRNA, via an A/U-rich sequence preceding stem-loop **b**. This binding causes the mRNA to adopt a structure in which stem-loop **b**, which follows the Hfq-binding site, is opened out to give a large loop containing the translation start codon, which lies within the sequence complementary to RyhB. The stem of stem-loop **b** starts with the ribosome-binding site. In conditions of iron deficiency (Fur inactivated), RyhB is produced and is stabilised by binding to Hfq. RyhB interacts with *sodB* mRNA by base pairing in the region containing the complementary sequence. This base pairing both modifies the structure of the RNA molecule and blocks translation. Changes in the structure of stem-loop **b** may lead to the release of Hfq. The block of translation and the structural change render the RNA molecule susceptible to RNase cleavages. Numbering starts at the transcription start site. The translation start site of *sodB* is indicated by an arrow. Hfq-binding sites are shown in red, and sequences complementary between *sodB* and RyhB are shown in green. Regions affected by Hfq binding are shown in bold.

sdhD mRNA–RyhB interaction is unknown, it seems to be different from that underlying the *sodB* mRNA–RyhB interaction. The sequence complementary to the *sdhD* mRNA is located in stem-loops 1 and 2 of RyhB, and is very long (up to 34 nucleotides), although there are some mismatches. In contrast, there are only 9 complementary nucleotides between RyhB and *sodB*, located in a different area, on stem-loop 2 of RyhB. Complexes form between RyhB and *sdhD* mRNA even in the absence of Hfq, suggesting that the *in vivo* requirement of Hfq for RyhB-mediated regulation of *sdhD* mRNA expression may be limited to stabilising RyhB.

It has been reported that RyhB of various bacteria all have a highly conserved core sequence, corresponding to loop 2; the structures of the 5′ and 3′ ends are conserved, but not the sequence (Massé and Gottesman, 2002). The site of interaction between RyhB and the *sodB* mRNA lies just within this conserved sequence. The 9 nucleotides complementary between RyhB and *sodB* are identical in *E. coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Vibrio cholerae* and

Yersinia pestis. Putative Hfq-binding sites (A/U-rich regions) have also been identified in the region following stem-loop 2 in RyhB from these bacteria, although the size of this region varies from 4 (*Y. pestis*) to 9 nucleotides (*V. cholerae*). Thus, the mode of *sodB* regulation by RyhB might be similar in these bacteria.

Role of Hfq in Fur (RyhB)-mediated degradation of sodB mRNA

Previous studies showed that expression of RyhB (*fur* mutant) results in rapid degradation of *sodB* mRNA (Dubrac and Touati, 2000). What is it that makes *sodB* mRNA susceptible to degradation upon pairing with RyhB? Degradation is probably triggered by the blocking of translation caused by the annealing of RyhB to the translation start site region. Massé *et al* (2003) recently showed that *sodB* and RyhB were broken down in a coupled process. As the Hfq and RNase E recognition sites are both single-stranded A/U-rich sequences, they suggested that Hfq binding directly blocks access by RNase E to RyhB, and that base pairing relieves this block by some still unclear process. Consistent with this, RyhB RNA is very unstable in the *hfq* mutant, and this depends on RNase E. Our data are not consistent with a similar model for *sodB* mRNA degradation. Unlike RyhB, the *sodB* mRNA is similarly stable in the presence or absence of Hfq (Figure 6B). And the stability of *sodB* mRNA in the absence of Hfq is no longer affected by the production of RyhB (*fur hfq* mutant; Figure 6B). Thus, the A/U-rich Hfq-binding site on *sodB* mRNA is not a target for RNase E in the absence of Hfq, despite it having a site similar to the sequence recognised by RNase E, suggesting that degradation is initiated elsewhere.

It had been assumed that Fur-RyhB regulation makes it possible to spare iron in conditions of iron starvation, by decreasing the production of iron-containing proteins. Not all iron proteins appear to be regulated by RyhB. So why is FeSOD regulated in this way? The answer probably lies in the abundance of this protein. Superoxide dismutase is required for survival in aerobic conditions, unlike other RyhB-regulated proteins, such as iron storage proteins, that are not needed in iron starvation. The decrease in FeSOD in the *fur* mutant is always offset by the Fur-dependent induction of MnSOD (Tardat and Touati, 1991, 1993). Very few studies have focused on the other genes regulated by RyhB. Such studies will show whether the Hfq targeting of *sodB* mRNA for RyhB repression is a general mechanism for regulation by RyhB or whether it is specific to *sodB* mRNA. If this mechanism should prove to be specific for *sodB* mRNA, then future studies should aim to uncover the reason for this specificity.

Materials and methods

Bacterial strains, phages and plasmids

The bacterial strains, phages and plasmids used in this study are listed in Table I. All the bacterial strains used are *E. coli* K-12 derivatives. Basic genetic manipulations were carried out according to standard procedures (Miller, 1992).

Construction of specific strains

Mutation of the A/T-rich region, such that sequence AAATTAA-TAATAAA was replaced by ACTGCAGCAATAAA, was carried out as follows: two PCR fragments were synthesized, one with the primers sodB12/sodB-AT-mut-rev and the other with primers sodB-AT-mut-for/sodB_140_trd (see http://www2.ijm.jussieu.fr/touati/ for all oligonucleotides used in this study). The two fragments were digested with *Pst*I and ligated together. The resulting fragment, $sodB_{AT}$, was used as a template for *in vitro* experiments and for fusion constructs.

Construction of Φ(sodB-lacZ) translational fusions

Fragments amplified by PCR with sodB12 and sodB_140_trd primers, from wild-type *sodB* and mutant $sodB_{AT}$ templates, were digested with *Eco*RI and *Bam*HI, ligated between the corresponding sites of pRS414 and transferred to the chromosome, as previously described, generating $(sodB\text{-}lacZ)_{19}$ (QC 6110) and $(sodB\text{-}lacZ)_{18}$ (QC 6108), respectively (Compan and Touati, 1993; Dubrac and Touati, 2000). Transcriptional fusion $(sodB\text{-}lacZ)_1$ was by insertion of fragment *Eco*RI–*Sna*BI from *sodB* into *Eco*RI–*Sma*I sites of pRS415. Fusions were checked by DNA sequencing, after amplification by PCR of the chromosomal DNA region from a single colony. We introduced *fur*::*kan*, *hfq*::*cat*, *ryhB*::*cat* mutations by PI transduction, as described previously (Compan and Touati, 1993). Insertion into the *hfq* mutant might have a polar effect on the downstream gene. We confirmed by complementation experiments with plasmids pTX349 and pTX367 and assays with strains carrying *hfq1*::Ω and *hfq2*::Ω mutations (Tsui *et al*, 1994) that the effects described in the Results section were due to the *hfq* mutation (data not shown).

Media, growth conditions, β-galactosidase assays and measurements of RNA stability

Cells were grown in Luria–Bertani (LB) medium at 37°C with shaking at 200 rpm. The following antibiotics were added as required: ampicillin (50 μg/ml), kanamycin (40 μg/ml) and chloramphenicol (20 μg/ml). β-Galactosidase activity was assayed as previously described (Compan and Touati, 1993). RNA stability was measured as described previously (Dubrac and Touati, 2000).

Templates for in vitro transcription

All templates for *in vitro* transcription were generated by PCR with forward primers containing the T7 promoter sequence. The Shine–Dalgarno sequence was mutated such that the sequence AGGAG was replaced by CTGC, destabilising the palindromic sequence (Figure 2A). This mutation was generated as described for $sodB_{AT}$, but using the primers sodB12/sodB-SD-mut-for and sodB-SD-mut-rev/sodB-140-trd. Again, the two fragments were digested with *Pst*I and ligated together. The resulting fragment, $sodB_{SD}$, was amplified with a forward primer containing the T7 promoter sequence and used as a template for *in vitro* transcription.

Gel mobility shift assays

RNA labelled with [α-^{32}P]UTP was produced by *in vitro* transcription with T7 RNA polymerase (Promega). RNA was purified on an 8% polyacrylamide/8 M urea gel and eluted in 0.5 M ammonium acetate, 1 mM EDTA and 0.1% SDS. The transcripts were collected by ethanol precipitation and suspended in 10 mM Tris–HCl, pH 8.5. Large amounts of unlabelled transcripts for competition experiments were generated with the T7 Megashortscript Kit (Ambion). For all competitive binding reactions, 1 fmol of the labelled transcript, 1 μg of yeast RNA, 5 pmol of unlabelled RNA and 0.1 pmol of purified Hfq (kindly provided by the team of P Régnier, IBPC, Paris) were mixed in 10 μl of 1 × binding buffer (50 mM Tris–HCl (pH 7.5), 250 mM NH_4Cl, 1 mM EDTA, 5% glycerol, 0.1% Triton X-100, 0.01% bromophenol blue). The samples were incubated at 37°C for 5 min and analysed on a 5% native polyacrylamide gel run in 1 × TBE at 4°C.

Minimal binding assays

$sodB_{1-148}$ or RyhB transcripts were radioactively labelled at the 5′ end with [γ-^{32}P]ATP and T4 Kinasemax labelling kit (Ambion) or at the 3′ end with [α-^{32}P]pCp and T4 RNA ligase (Ambion). The labelled fragments were purified by polyacrylamide gel electrophoresis as described above. In all, 5 μl (1 pmol) of the purified transcripts was treated with 0.5 μl of alkaline buffer (0.5 M NaOH, 10 mM EDTA) at 95°C for 45 s and immediately neutralised by adding 0.5 μl of acid buffer (0.5 M HOAc). The hydrolysed RNA fragments were collected by ethanol precipitation and incubated with 1 or 7 pmol Hfq and 1 μg of yeast RNA in 1 × binding buffer. The bound and unbound RNAs were separated on a native polyacrylamide gel, as described above. The fractions were

Table I Bacterial strains, phages and plasmids used in this study[a]

Strain, phage or plasmid	Genotype or relevant characteristics	Source or reference
E. coli strains		
MG1655	F$^-$ λ$^-$ *rph-1*	Genetic Stock Center
HAT10	F$^-$ *ara* Δ*(lac-pro)thi hfq10*::*cat*	Wachi *et al* (1999)
EM1238	MG1655 Δ*X74lac* Δ*ryhB1*::*cat*	Massé and Gottesman (2002)
QC1732	F$^-$ Δ*lacU169 rpsL fur*::*kan*	Compan and Touati (1993)
QC2461	MG1655 Δ*lac(IZ)*	Dubrac and Touati (2000)
QC2550	QC2461 Φ(*sodB-lacZ*)$_1$	This work
QC2567	QC 2550 Δ*fur*::*kan*	This work
QC2700	QC2461 Φ(*sodB-lacZ*)$_3$	Dubrac and Touati (2000)
QC2704	QC 2700 Δ*fur*::*kan*	Dubrac and Touati (2000)
QC6108	QC2461 Φ(*sodB-lacZ*)$_{18}$	This work
QC6109	QC6108 Δ*fur*::*kan*	This work
QC6110	QC2461 Φ(*sodB-lacZ*)$_{19}$	This work
QC6111	QC6110 Δ*fur*::*kan*	This work
QC6112	QC6110 *hfq10*::*cat*	This work
QC6114	QC6111 *hfq10*::*cat*	This work
Phages		
λRS45		Simons *et al* (1987)
λTG1	λRS45 recombinant carrying Φ(*sodB-lacZ*)$_{18}$	This work
λTG2	λRS45 recombinant carrying Φ(*sodB-lacZ*)$_{19}$	This work
λDT12-2	λRS45 recombinant carrying Φ(*sodB-lacZ*)$_{17}$	This work
λDT12-3	λRS45 recombinant carrying Φ(*sodB-lacZ*)$_{20}$	This work
Plasmids		
pRS414		Simons *et al* (1987)
pRS415		Simons *et al* (1987)
pHS1-8	pBR322 derivative carrying the *sodB* region	Sakamoto and Touati (1984)
pTG1	pRS414 with fragment containing the sodB$_{(-800)-(+148)}$ promoter region with modifications in the A/T-rich region; (*sodB-lacZ*)$_{18}$	This work
pTG2	pRS414 with fragment containing the sodB$_{(-800)-(+148)}$ promoter region; (*sodB-lacZ*)$_{19}$	This work

[a]Described also on our website http://www2.ijm.jussieu.fr/touati/.

excised, eluted and precipitated with ethanol. They were then suspended in gel loading buffer (95% formamide, 18 mM EDTA, 0.025% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol) and separated on an 8% polyacrylamide/8 M urea sequencing gel run in 1 × TBE.

RNA footprinting

In total, 0.2 pmol of 5′ end-labelled sodB$_{1-148}$ or RyhB was incubated with (1 pmol) or without Hfq and 1 μg yeast RNA in 10 μl of 1 × structure buffer (10 mM Tris–HCl (pH 7.0), 100 mM KCl, 10 mM $MgCl_2$) at 37°C for 5 min. Subsequently, 1 μl of RNase A (1 ng), RNase T_1 (0.1 U), RNase V_1 (0.001 U) or RNase I (0.1 U) (all from Ambion) was added and the incubation was continued for 5 min. The reaction was stopped by adding 20 μl of inactivation buffer (Ambion), and the transcripts were precipitated, suspended in gel loading buffer and analysed on an 8% polyacrylamide/8 M urea sequencing gel run in 1 × TBE.

The same experiments were used to analyse the RNA/RNA interaction, except that unlabelled sodB$_{1-148}$, full-length *sodB* mRNA or RyhB RNA was added (in 50-fold molar excess).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank E Hajnsdorf and M Folichon for donating purified Hfq protein, E Massé and S Gottesman for communicating their results before publication, and S Gottesman for critical and stimulating comments on our work. TG was supported by a fellowship from the Swiss National Science Foundation.

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