Functional replacement of the *Escherichia coli* *hfq* gene by the homologue of *Pseudomonas aeruginosa*

Elisabeth Sonnleitner, Isabella Moll and Udo Bläsi

Author for correspondence: Udo Bläsi. Tel: +43 1 4277 54609. Fax: +43 1 4277 9546.
e-mail: udo.blaesi@univie.ac.at

Institute of Microbiology
and Genetics, Vienna
Biocentre, Dr Bohrgasse 9,
1030 Vienna, Austria

The 102 aa Hfq protein of *Escherichia coli* (Hfq_{Ec}) was first described as a host factor required for phage Q β replication. More recently, Hfq was shown to affect the stability of several *E. coli* mRNAs, including *ompA* mRNA, where it interferes with ribosome binding, which in turn results in rapid degradation of the transcript. In contrast, Hfq is also required for efficient translation of the *E. coli* and *Salmonella typhimurium* *rpoS* gene, encoding the stationary σ factor. In this study, the authors have isolated and characterized the Hfq homologue of *Pseudomonas aeruginosa* (Hfq_{Pa}), which consists of only 82 aa. The 68 N-terminal amino acids of Hfq_{Pa} show 92 % identity with Hfq_{Ec}. Hfq_{Pa} was shown to functionally replace Hfq_{Ec} in terms of its requirement for phage Q β replication and for *rpoS* expression. In addition, Hfq_{Pa} exerted the same negative effect on *E. coli* *ompA* mRNA expression. As judged by proteome analysis, the expression of either the plasmid-borne *hfq*_{Pa} or the *hfq*_{Ec} gene in an *E. coli* Hfq[−] RpoS[−] strain revealed no gross difference in the protein profile. Both Hfq_{Ec} and Hfq_{Pa} affected the synthesis of approximately 26 RpoS-independent *E. coli* gene products. These studies showed that the functional domain of Hfq resides within its N-terminal domain. The observation that a C-terminally truncated Hfq_{Ec} lacking the last 27 aa [Hfq_{Ec(75)}] can also functionally replace the full-length *E. coli* protein lends further support to this notion.

Keywords: global regulator, Hfq, *ompA*, *rpoS*

INTRODUCTION

Escherichia coli host factor I (HF1/Hfq), the product of the *hfq* gene, was first described as a factor required for phage Q β replication (de Fernandez *et al.*, 1968). More recent experiments have suggested that Hfq stimulates Q β replication by melting out the 3' end of Q β plus-strand RNA, which appears to facilitate access of the replicase (Schuppli *et al.*, 1997).

Hfq has been reported to positively regulate the *rpoS* gene encoding the σ^S subunit of RNA polymerase (Brown & Elliot, 1996; Muffler *et al.*, 1996). It has been suggested that Hfq activates *rpoS* translation by altering the secondary structure, sequestering its RBS (Brown & Elliot, 1997; Cunnings *et al.*, 1998; Muffler *et al.*, 1996).

However, several other factors, including HNS and two small RNAs (DsrA and OxyS), are also involved in *rpoS* mRNA translation (Sledjeski *et al.*, 1996, 2001; Wassarman *et al.*, 2001; Zhang *et al.*, 1998). Whereas DsrA RNA seems to stimulate the translation of *rpoS* by pairing with the mRNA and melting the secondary structure (Majdalani *et al.*, 1998), OxyS RNA represses *rpoS* translation by binding to Hfq (Zhang *et al.*, 1998). Although OxyS RNA does not prevent the binding of Hfq to the *rpoS* mRNA it apparently abrogates the stimulatory effect of Hfq on *rpoS* translation.

Hfq has been implicated in affecting the stability of the mRNAs of *mutS*, *miaA*, *hfq* (Tsui *et al.*, 1997) and *ompA* (Vytvytska *et al.*, 1998). Vytvytska *et al.* (2000) have shown that Hfq binds to the 5'-untranslated region (UTR) of *ompA* in the vicinity of the RBS, preventing translation and thereby indirectly subjecting the mRNA to degradation.

The *E. coli* Hfq protein (Hfq_{Ec}) is heat-stable and consists of 102 aa. Immunofluorescence microscopy

Abbreviations: Hfq_{Ec}, Hfq protein of *Escherichia coli*; Hfq_{Ec(75)}, C-terminally truncated Hfq_{Ec} lacking the last 27 aa; Hfq_{Pa}, Hfq homologue of *Pseudomonas aeruginosa*; UTR, untranslated region.

indicated that the majority of Hfq_{Ec} is present in the cytosol and that it is most likely associated with the translation machinery; only a minor fraction appears to be associated with the nucleoid (Azam *et al.*, 2000). Mutations in the *E. coli* *hfq* gene (*hfq*_{Ec}) caused pleiotropic effects. The insertion of an Ω cassette at the beginning of *hfq* resulted in a decreased growth rate, an increase in cell size, osmo-sensitivity and an increased sensitivity to UV light, and it also affected the supercoiling of plasmids (Tsui *et al.*, 1994). Furthermore, a *hfq* null-mutant did not synthesize glycogen, was starvation and multiple-stress sensitive and, as expected from its positive effect on *rpoS* translation, showed a down-regulation of RpoS-regulated genes (Muffler *et al.*, 1997). Taken together with the above-described effects on specific genes, these results suggested that Hfq acts as a global regulator involved in the regulation of RpoS-dependent and RpoS-independent genes. Moreover, Hfq homologues have been reported to stimulate synthesis of the heat-stable enterotoxin in *Yersinia enterocolitica* (Nakao *et al.*, 1995) and of the NifA protein in *Azorhizobium caulinodans* (Kaminski *et al.*, 1994). Also, the Hfq homologue of *Brucella abortus* appears to be a major determinant of virulence in mice (Robertson & Roop, 1999).

As an opportunistic human pathogen *Pseudomonas aeruginosa* causes serious infections in immunocompromised hosts, but rarely infects healthy individuals (Bodey *et al.*, 1983). It has been previously shown that RpoS of *P. aeruginosa* is required for the expression of severe-stress-resistance genes as well as for the expression of several virulence genes (Suh *et al.*, 1999). Since Hfq is required in both *E. coli* and *Salmonella typhimurium* for the efficient translation of *rpoS*, we were interested to determine whether a Hfq homologue is present in *P. aeruginosa*. Here, we report the isolation of the *P. aeruginosa* *hfq* gene (*hfq*_{Pa}). We also show that the Hfq protein of *P. aeruginosa* (Hfq_{Pa}) can functionally replace Hfq_{Ec}.

METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table 1. *E. coli* IM1101 was constructed by transducing *rpoS*-354::Tn10 from *E. coli* RH90 to *E. coli* AM111, using phage P1 and standard procedures (Miller, 1972). Unless indicated otherwise, bacterial cultures were grown at 37 °C in Luria-Bertani (LB) medium (Miller, 1972), supplemented with the appropriate antibiotics. Antibiotics were added to the following final concentrations: 100 µg ampicillin ml⁻¹, 25 µg kanamycin ml⁻¹, 10 µg chloramphenicol ml⁻¹, 15 µg gentamicin ml⁻¹ and 20 µg tetracycline ml⁻¹.

Construction of plasmids used in this study. pESE102, pESE75 and pESP82 (Table 1) were constructed as follows. The *hfq*_{Ec} gene [nucleotide -16 to +310 with regard to the A (position +1) of the start codon] was amplified by PCR using pHFQ607 and the forward primer E15 (5'-TTTTTTGAATTCGGATCGTGAGGAAAAGAGAGAATGGCTAAGGGG-3'), containing a *Bam*HI site (bold), together with the reverse primer

5'-CCCGAATTCTTATTCGGTTTCGCTGTCC-3', containing an *Eco*RI site (bold). The DNA fragment was cleaved with *Bam*HI and *Eco*RI, and then cloned into the corresponding sites of pUC19, resulting in pESE102.

Plasmid pESE75 was constructed by first amplifying a fragment carrying the 3'-terminally truncated *hfq*_{Ec} gene [nucleotide -16 to +225; *hfq*_{Ec(75)}] by means of PCR using pHFQ607 and the forward primer E15 together with the reverse primer 5'-TTTTTTTGGATCCCTGCAGttactaGGCGTTGTACTGTGATGAG-3' containing a *Pst*I site (bold) and two stop codons (lower case). This PCR fragment was then inserted into the *Bam*HI-*Pst*I sites of vector pUC18.

The putative *hfq*_{Pa} gene (position 5548396-5548644 of the *P. aeruginosa* genomic sequence, www.pseudomonas.com; accession no. AE004091) was amplified by PCR from *P. aeruginosa* PAO1 genomic DNA using the forward primer X14 (5'-TTTTTTTGGATCCCTATTCGACTAC-3'), containing a *Bam*HI site (bold), and the reverse primer Y14 (5'-TTTTTTTCTGCAGCCTGTCCACCACC-3'), containing a *Pst*I site (bold). The *hfq*_{Pa} gene fragment (nucleotides -46 to +296) was digested with *Bam*HI and *Pst*I, and then cloned into the corresponding sites of pUC18, resulting in pESP82.

Plasmids pDLE102, pDLE75 and pDLP82 are derivatives of pACYC184 and were constructed as follows. pESE102, pESE75 and pESP82, respectively, were cleaved with *Pvu*II. The resulting fragments, containing the *lac* promoter and the *hfq*_{Ec}, *hfq*_{Ec(75)} or *hfq*_{Pa} gene, were inserted into the *Eco*RV site of pACYC184, resulting in pDLE102, pDLE75 and pDLP82, respectively.

Plasmid pESPGm is a derivative of pESP82. The gentamicin cassette, *aacC1*, from pUCP24 was amplified by PCR using the primer pair B15 (5'-TTTTTTTGTATTCGGTACCTCTAGACCAGCGGCACCAGCGGC-3') and C15 (5'-TTTTTTTGTATTCGGTACCGCGGCGTTGTGAC-3'); both of these primers contained a *Kpn*I site (bold). The PCR fragment was digested with *Kpn*I and cloned into the corresponding site of plasmid pUC19. The resulting pUC19 derivative was then cleaved with *Pvu*II and *Hinc*II, and the fragment was inserted into the *Hinc*II site of pESP82 to generate pESPGm, which bore the insertionally inactivated *hfq*_{Pa} gene.

The *ompA-lacZ* fusion plasmid, pIMZ, was constructed as follows. A constitutive *lac* promoter without operator sites was amplified by PCR from pUHE21-2 using the forward primer C10 (5'-AAATCTAGAATTCCTTTTCGTCTTCACTCGAG-3'), containing a *Xba*I site (bold), and the reverse primer C12 (5'-AAAAAAGAATTCATCTAAGTATCATGTATTATCCG-3'), containing an *Eco*RI site (bold). The resulting fragment was cleaved with both *Eco*RI and *Xba*I and ligated to a ~1300 bp *Eco*RI-*Hind*III fragment isolated from pKSO325, containing the full-length *ompA* gene. This fragment was used to amplify the constitutive *lac* promoter together with the 5'-UTR of *ompA* up to nucleotide +69 by PCR with the forward primer C10 and the reverse primer E12 (5'-AAAAAAGGATCCGGAGCGGCCTGCGCTAGGG-3'), containing a *Bam*HI site (bold). This fragment was then cloned into the *Xba*I and *Bam*HI sites of pRB381 in-frame with *lacZ*, resulting in pRBompA-*lacZ*. This plasmid was then digested with *Eco*RI and *Bam*HI. The *Eco*RI-*Bam*HI fragment was cloned into plasmid pUHE21-2 downstream of the *lac* promoter. The resulting pUHE21-2 derivative was then cleaved with *Xba*I and *Bam*HI, and the fragment containing the *lac* promoter and the 5' end of *ompA* was inserted into the

Table 1. Bacterial strains and plasmids

Strains/plasmids	Genotype/relevant features	Source/reference
<i>P. aeruginosa</i>		
PAO1		Holloway (1955)
<i>E. coli</i>		
AM111	MC4100 <i>hfq-1::</i> Ω	Muffler <i>et al.</i> (1996)
AM111F'	AM111F' <i>proAB lacI^q lacZΔM15 Tn10; Tc^R</i>	Lab. stock
IM1101	MC4100 <i>hfq-1::</i> Ω <i>rpoS-354::</i> Tn10	This study
RH90	MC4100 <i>rpoS-354::</i> Tn10	Lange & Hengge-Aronis (1991)
Plasmids		
pUC18	<i>colE1 ori; Amp^R</i>	Yanisch-Perron <i>et al.</i> (1985)
pUC19	<i>colE1 ori; Amp^R</i>	Yanisch-Perron <i>et al.</i> (1985)
pESE102	pUC19 with <i>hfq_{Ec}</i> cloned under P _{<i>lac</i>} control	This study
pESE75	pUC18 with <i>hfq_{Ec(75)}</i> cloned under P _{<i>lac</i>} control	This study
pESP82	pUC18 with <i>hfq_{Pa}</i> cloned under P _{<i>lac</i>} control	This study
pESPGm	pESP82 with <i>hfq_{Pa}::aacC-1</i>	This study
pUCP24	pUC18 derivative carrying an <i>aacC-1</i> cassette; Gm ^R	West <i>et al.</i> (1994)
pKSO325	Harbours <i>E. coli ompA</i>	Ried <i>et al.</i> (1994)
pOU251	Single-copy-number vector at 28 °C with <i>oriR; Amp^R</i>	J. Jakobsen, University of Vienna
pRB381	<i>E. coli-B. subtilis</i> shuttle vector; Amp ^R	Brückner (1992)
pRB- <i>ompA-lacZ</i>	pRB381 with an <i>ompA-lacZ</i> fusion gene under the control of a constitutive P _{<i>lac</i>}	This study
pIMZ	pOU251 with an <i>ompA-lacZ</i> fusion gene under control of P _{<i>lac</i>}	This study
pACYC184	P15A <i>ori; Tet^R, Kan^R</i>	Rose (1988)
pDLE102	pACYC184 with <i>hfq_{Ec}</i> cloned under P _{<i>lac</i>} control	This study
pDLE75	pACYC184 <i>hfq_{Ec(75)}</i> cloned under P _{<i>lac</i>} control	This study
pDLP82	pACYC184 <i>hfq_{Pa}</i> cloned under P _{<i>lac</i>} control	This study
pHFQ607	<i>E. coli hfq</i> gene cloned under transcriptional control of a phage T7 promoter	Kajitani <i>et al.</i> (1994)
pUHE21-2	<i>colE1 ori; Amp^R</i>	Lanzer & Bujard (1988)

corresponding sites of the single-copy, (28 °C)/transcriptional-fusion vector pOU251 in-frame with *lacZ*, resulting in pIMZ.

Qβ plating assay. Overnight cultures (100 µl) of *E. coli* AM111F' harbouring pUC18, pESE102, pESE75 or pESP82 were diluted in 5 ml LB and the corresponding *hfq* genes were induced by adding IPTG to a final concentration of 3 mM. The cultures were grown at 37 °C until they reached an OD₆₀₀ of 0.4. Then 200 µl aliquots of the cultures and 10 µl of the Qβ phage dilutions were added to 3 ml top agar. The agar was poured onto LB agar plates supplemented with the appropriate antibiotics and 3 mM IPTG. The p.f.u. ml⁻¹ values were calculated from triplicate assays.

β-Galactosidase assay. *E. coli* AM111F' containing pIMZ and one of the pACYC-*hfq* derivatives (pDLE102, pDLE75, pDLP82 or pACYC184) was incubated at 28 °C. At an OD₆₀₀ of 0.3, the pIMZ-encoded *ompA-lacZ* gene and the respective *hfq* genes were induced by the addition of IPTG (3 mM). The β-galactosidase activity (Miller, 1972) was determined from triplicate samples at 28 °C, to maintain a single copy of pIMZ.

Western blot analysis. Cultures of AM111F', harbouring pESP82, pESPGm, pESE102, pESE75 or pUC18, and strain RH90 were grown at 37 °C until they reached an OD₆₀₀ of 0.4, at which time IPTG was added to a final concentration of 3 mM. At an OD₆₀₀ of 0.8 equal amounts of cells were withdrawn and boiled in Laemmli buffer. The proteins were

separated on 12 % SDS-polyacrylamide gels (Laemmli, 1970) and then transferred to a nitrocellulose membrane. The blots were blocked with 5 % dry milk in TBS (8 g NaCl l⁻¹, 0.2 g KCl l⁻¹ and 3 g Tris-base l⁻¹ in water, pH 7.5) and then probed with anti-RpoS antibodies (kindly provided by Dr F. Norel, Pasteur Institute, Paris). The antibody-antigen complex was visualized with goat-anti-rabbit immunoglobulin alkaline-phosphatase-conjugated antibodies (Sigma) using the chromogenic substrate nitro blue tetrazolium [2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)-ditetrazolium chloride; BIOMOL] and BCIP (5-bromo-4-chloro-3-indolyl phosphate) *p*-toluidine salt (BIOMOL) in alkaline phosphatase buffer (10 mM NaCl, 5 mM MgCl₂, 100 mM Tris/HCl, pH 9.5). The quantification of the protein bands on the Western blots was performed with ImageQuant software (Molecular Dynamics, version 3.3).

Proteome analysis. *E. coli* IM1101 harbouring pUC18, pESE102 or pESP82 was grown in M9 minimal medium containing 0.2 % (v/v) glycerol to an OD₆₀₀ of 0.8 and then labelled with 143 pM L-[³⁵S]methionine (Amersham Pharmacia Biotech; >37 TBq mM⁻¹) for 10 min. Total cellular protein extracts were analysed by two-dimensional gel electrophoresis. Equal amounts of cell material were dissolved in lysis buffer (8 M urea, 4 %, w/v, CHAPS and 40 mM Tris-base) and the cells were disrupted by repeated freezing in liquid N₂ and thawing at 37 °C. For the first dimension the Immobiline Dry strip pH 3–10 (18 cm) (Amersham Pharmacia Biotech)

was used with the following IEF programme: 12 h rehydration, 1 h 500 V, 1 h 1000 V, 4 h 8000 V (IPGphor isoelectric focusing system). Resolution in the second dimension was performed on 12.5% SDS polyacrylamide gels for 15 min at 10 mA and then for 5 h at 20 mA. Buffers and conditions were used according to the manufacturer's instructions. The gels were dried and then exposed to a Molecular Dynamics Phosphor Imager screen and analysed with PDQuest software (Bio-Rad).

Computer analysis. The protein sequence of Hfq_{Pa} was revealed by comparing the protein sequence of Hfq_{Ec} with the proteins predicted to be encoded by the *P. aeruginosa* genome (Stover *et al.*, 2000), using the sequence-alignment algorithm BLASTP (Altschul *et al.*, 1990). Preliminary sequence data were obtained from the Institute of Genomic Research (TIGR; <http://www.tigr.org>). Multiple alignments were done with CLUSTAL W (Thompson *et al.*, 1994) and visualized using BOXSHADE. The following (putative) Hfq sequences were compared: *Pseudomonas aeruginosa* (accession no. AE004091), *Pseudomonas putida* (NC002947), *Pseudomonas syringae* (NC002949), *Escherichia coli* (U00005), *Salmonella typhimurium* (U48735), *Yersinia enterocolitica* (D28762), *Pectobacterium carotovorum* (AF039142), *Haemophilus influenzae* (U32724), *Shigella flexneri* (AB000785), *Aquifex aeolicus* (AE000674), *Brucella abortus* (AF154075), *Acidithiobacillus ferrooxidans* (NC002923), *Azorhizobium caulinodans* (X76450), *Bacillus halodurans* (BA000004), *Bacillus subtilis* (AL009126) and *Clostridium acetobutylicum* (AE001437). The name of the funding agency for each of the different bacterial genome projects can be found on the TIGR website.

RESULTS AND DISCUSSION

Sequence similarity between Hfq_{Pa} and (putative) Hfq homologues from other bacteria

After identification of the putative Hfq_{Pa} protein (see Methods), we performed a search for homologous proteins using the sequence alignment algorithm BLASTP. As shown in Fig. 1, Hfq_{Pa} shows considerable amino acid similarity to previously identified Hfq homologues from other Gram-negative bacteria (73 to 96%) (Brown & Elliott, 1996; Chuang *et al.*, 1999; Deckert *et al.*, 1998; Durand *et al.*, 1997; Fleischmann *et al.*, 1995; Kajitani & Ishihama, 1991; Kaminski *et al.*, 1994; Nakao *et al.*, 1995; Robertson & Roop, 1999), whereas lower amino acid similarity (41 to 45%) was found with putative Hfq homologues from three Gram-positive bacteria. The multiple alignment (Fig. 1) revealed a high conservation of the N-terminal amino acids. There is considerable variation in the C-terminal amino acids, suggesting that the functional domain of Hfq resides within the first 70 aa. This suggestion is supported by the fact that insertion of an Ω cassette approximately in the middle of *hfq*_{Ec} (nucleotide +117, after codon 39) rendered the protein inactive, whereas insertion of the cassette at the 3' end of the gene (nucleotide +236, after codon 78) did not affect the activity of the protein (Tsui *et al.*, 1994). In addition, further support for this assertion stems from the fact that the 82 aa Hfq_{Pa} is only conserved within its first 68 aa. Finally, in the Hfq homologues of the different Gram-negative bacteria, and to a somewhat reduced

extent in that of Gram-positive bacteria, positively charged amino acids are found at invariant positions within the first 70 aa (Fig. 1), suggesting their functional importance in nucleic acid binding.

Both Hfq_{Pa} and Hfq_{Ec(75)} can functionally replace Hfq_{Ec} in phage Q β replication

Because of the identity between their N-terminal amino acids, we asked whether *hfq*_{Pa} could functionally replace *hfq*_{Ec}. Therefore, pESE102 and pESP82 harbouring *hfq*_{Ec} and *hfq*_{Pa}, respectively, were constructed. The Hfq proteins of *E. coli*, *S. typhimurium*, *Y. enterocolitica* and *Pectobacterium carotovorum* are nearly identical up to amino acid 75 (Fig. 1). pESE75, encoding the first 75 aa (*hfq*_{Ec(75)}), was therefore created with the aim of determining whether the C terminus of Hfq_{Ec} was functionally irrelevant. The expression of *hfq*_{Ec}, *hfq*_{Pa} and *hfq*_{Ec(75)} was verified by Western blot analysis with antibodies against Hfq_{Ec}. As judged by quantitative Western blotting, the apparent level of production of Hfq_{Pa} was reduced by approximately threefold when compared to that of both Hfq_{Ec} and Hfq_{Ec(75)} (data not shown), which could result either from a reduced cross-reactivity with the antibodies raised against Hfq_{Ec} or from a reduced rate of expression of *hfq*_{Pa} in *E. coli*.

Since Hfq is essential for phage Q β replication (Carmichael *et al.*, 1975; de Fernandez *et al.*, 1972), we tested the functionality of Hfq_{Pa} and Hfq_{Ec(75)} in its replication. When compared to strain AM111F'-(pESE102) (1.1×10^{10} p.f.u. ml⁻¹), no significant difference in the p.f.u. ml⁻¹ value was detected with strain AM111F'-(pESE75) (1.0×10^{10} p.f.u. ml⁻¹) or strain AM111F'-(pESP82) (1.0×10^{10} p.f.u. ml⁻¹), whereas phage Q β was unable to replicate in the control strains AM111F' and AM111F'-(pESPGm), the latter of which carries the inactivated *hfq*_{Pa} gene (Table 1). Thus, the seemingly reduced Hfq_{Pa} levels present in strain AM111F'-(pESP82) were apparently not limiting for phage Q β replication.

Both Hfq_{Pa} and Hfq_{Ec(75)} affect the expression of *E. coli ompA* in a negative manner

It has been shown previously that Hfq_{Ec} binds to the 5' UTR of *E. coli ompA* mRNA, which results in a decreased rate of expression for *ompA* (Vytvytska *et al.*, 2000). To test whether the Hfq_{Pa} and the Hfq_{Ec(75)} protein exerted the same effect on the expression of *ompA*, expression of the pIMZ-encoded *ompA-lacZ* fusion was monitored in AM111F' co-transformed with pDLE102 (*hfq*_{Ec}), pDLE75 (*hfq*_{Ec(75)}), pDLP82 (*hfq*_{Pa}) or pACYC184. Both *ompA-lacZ* and *hfq* expression was induced at an OD₆₀₀ of 0.3. At an OD₆₀₀ of 0.6 the cells were harvested and the β -galactosidase activities were determined. When compared to Hfq_{Ec}, the presence of Hfq_{Pa} or Hfq_{Ec(75)} resulted in a similar decrease in the expression rate of the *ompA-lacZ* fusion (data not shown), demonstrating that *ompA* mRNA is also a target for negative regulation by Hfq_{Pa} and by Hfq_{Ec(75)}.

<i>P. aeruginosa</i>	1	---MSKGHSLODPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---TV
<i>E. coli</i>	1	---MAKGQSLQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---TV
<i>S. typhimurium</i>	1	---MAKGQSLQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---TV
<i>Y. enterocolitica</i>	1	---MAKGQSLQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---TV
<i>S. flexneri</i>	1	---MAKGQSLQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---TV
<i>H. influenzae</i>	1	---MAKGQSLQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---TV
<i>P. carotovorum</i>	1	---MAKGQSLQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---TV
<i>P. syringae</i>	1	---MSKGHSLODPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---TV
<i>P. putida</i>	1	---MSKGHSLODPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---TV
<i>A. ferrooxidans</i>	1	MAGQKGGVLODPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---NW
<i>A. aeolicus</i>	1	---MPYKLOESFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---GKQ
<i>B. abortus</i>	1	---MAEESQMLQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---RDGH
<i>A. caulinodans</i>	1	---MAEESQMLQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---RDGH
<i>B. halodurans</i>	1	---MKSSVNDQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---TEGK
<i>B. subtilis</i>	1	---MKPINKQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---SEEG
<i>C. acetobutylicum</i>	1	---MNNKSTMLQDPFLNLRKERVVSIYLVNGIKLQGGIESFDQFVILLKN---ESDG

<i>P. aeruginosa</i>	51	SQMVYKHAISTVVPSPVSRPVP-----SGDQPAEPQNA-----
<i>E. coli</i>	51	SQMVYKHAISTVVPSPVSHHSNAGGGTSSNYHGSNAQNTSAQDSEETE
<i>S. typhimurium</i>	51	SQMVYKHAISTVVPSPVSHHSNAGGGASNNYHGSNAQNTSAQDSEETE
<i>Y. enterocolitica</i>	51	SQMVYKHAISTVVPSPVSHHSNNPS-GSTNNYHGSNPSAPQPPQDSDDAE
<i>S. flexneri</i>	51	SQMVYKHAISTV-----
<i>H. influenzae</i>	51	SQMVYKHAISTVVPSPVSHHSNNH-----HTAPTEAVEVETQAE
<i>P. carotovorum</i>	51	SQMVYKHAISTVVPSPVSHHSNNP-----GGSNNYHGSNTTAQSSQADDAE
<i>P. syringae</i>	51	SQMVYKHAISTVVPSPVSRPVPSPATD-----ADGADAEPQNA-----
<i>P. putida</i>	51	SQMVYKHAISTVVPSPVSRPVPSPPTD-----SEHGDSPEQNA-----
<i>A. ferrooxidans</i>	54	SQMVYKHAISTVVPSPVSRPVP-----LPEAEGESSTEEEA-----
<i>A. aeolicus</i>	50	QTVYKHAISTVVPSPVSRPVP-----VPGG-----
<i>B. abortus</i>	53	SQMVYKHAISTVVPSPVSRPVP-----EGEEA-----
<i>A. caulinodans</i>	54	SQMVYKHAISTVVPSPVSRPVP-----DPTDEVASEKA-----
<i>B. halodurans</i>	52	QQVYKHAISTVVPSPVSRPVP-----TENEPS-----
<i>B. subtilis</i>	51	QQLVYKHAISTVVPSPVSRPVP-----LE-----
<i>C. acetobutylicum</i>	54	QMMYKHAISTVVPSPVSRPVP-----QPDDEE-----

Fig. 1. Sequence of Hfq_{Pa} and (putative) Hfq homologues from Gram-positive and -negative bacteria. Approximately the first 70 aa of the N-terminal of Hfq are highly homologous. Identical amino acids are highlighted black, whereas conservative exchanges are highlighted grey. Positively charged residues, at invariant positions, are indicated by an arrow.

Fig. 2. Both Hfq_{Pa} and Hfq_{Ec(75)} stimulate the expression of *rpoS* in *E. coli*. (a) Western blot analysis was carried out as described in Methods. Only the relevant section of the immunoblot showing the RpoS-specific band is depicted. Lanes 1–5 are visual representations of the RpoS levels in *E. coli* AM111F harbouring the following plasmids. Lanes: 1, pESE102 (*hfq_{Ec}*); 2, pESE75 (*hfq_{Ec(75)}*); 3, pESP82 (*hfq_{Pa}*); 4, pESPGm (*hfq_{Pa}::aacC1*); 5, pUC18 (control). Lane 6, RpoS⁻ strain RH90. No RpoS signal was detected for this strain. (b) Graphical representation of the results presented in (a). Quantification of the Western blot signals was done with the ImageQuant software. Values were normalized to the RpoS signal obtained from AM111F(pUC18), which was set at 1. Results represent data from triplicate experiments; the error bars represent SD.

Both Hfq_{Pa} and Hfq_{Ec(75)} stimulate the expression of *rpoS*

Previous studies by Muffler *et al.* (1996) and by Brown & Elliott (1996) have demonstrated that Hfq is required for the efficient translation of *rpoS* in *E. coli* and *S. typhimurium*, respectively. We therefore asked whether Hfq_{Pa} and Hfq_{Ec(75)} showed a similar positive effect on the translation of *rpoS* to that shown by Hfq_{Ec}. The *hfq_{Ec}*, *hfq_{Pa}* and *hfq_{Ec(75)}* genes, encoded by pESE102, pESP82 and pESE75, respectively, were induced in AM111F⁺ and the differences in the RpoS levels were determined by quantitative Western blotting. The induction of *hfq_{Ec}* and *hfq_{Ec(75)}* (Fig. 2a, lanes 1, 2 and Fig. 2b) resulted in a similar increase (~12-fold) in the RpoS concentration when compared to that present in the Hfq⁻ strains AM111F⁺(pESPGm) and AM111F⁺(pUC18) (Fig. 2a, lanes 4 and 5 and Fig. 2b) and RH90 (Fig. 2a, lane 6 and Fig. 2b). The expression of *hfq_{Pa}* (Fig. 2a, lane 3 and Fig. 2b) resulted in an approximately sevenfold increase in the RpoS concentration when compared to the control strains. Whether this results from the apparently reduced levels of Hfq_{Pa} present, when compared to those of Hfq_{Ec} or Hfq_{Ec(75)}, remains to be determined.

Hfq_{Pa} and Hfq_{Ec} show the same regulatory effects on RpoS-independent genes

Several studies have suggested that Hfq acts as global regulator, which affects the expression of RpoS-dependent as well as RpoS-independent genes (Muffler *et al.*, 1997; Tsui *et al.*, 1997; Vytvytska *et al.*, 2000). To test whether Hfq_{Pa} and Hfq_{Ec} show the same alteration

Fig. 3. Two-dimensional protein synthesis pattern of *E. coli* IM1101 bearing pUC18 (a), pESE102 (b) and pESP82 (c), respectively. Total cellular protein extracts were analysed. The pH gradient in the first dimension ranged from 3 to 10 (from left to right). Proteins whose synthesis was decreased in the presence of Hfq are depicted by solid arrowheads; those proteins whose synthesis was turned off in the presence of Hfq are indicated by open arrowheads. The proteins whose synthesis was enhanced in the presence of Hfq are indicated by solid arrows. Proteins which were absent in IM1101(pUC18) and present in the strains containing the plasmid-encoded Hfq_{Pa} or Hfq_{Ec} are indicated by open arrows.

Table 2. Regulatory effects by Hfq on the synthesis of RpoS-independent genes

Putative protein	pI Value	Molecular mass (kDa)
Increased protein synthesis in the presence of Hfq (solid arrows in Fig. 3)		
(e) Malate dehydrogenase	5.55	35.5
(g) 2,3,4,5-Tetrahydropyridine-2-carboxylate N-succinyltransferase	5.51	31.7
(h) –	5.58	32.7
(i) –	5.21	32.8
(q) Adenylate kinase	5.60	22.5
Induced protein synthesis in the presence of Hfq (open arrows in Fig. 3)		
(c) D-Aspartate aminotransferase	5.40	42.0
(j) –	5.32	32.4
(k) –	5.41	32.4
(r) –	5.40	20.1
Decreased protein synthesis in the presence of Hfq (solid arrowheads in Fig. 3)		
(a) Periplasmic oligopeptide-binding protein	5.93	56.1
(b) Elongation factor–Tu (EF–Tu)	5.32	44.6
(f) Elongation factor–Ts (EF–Ts)	5.15	33.6
(l) Chemotaxis protein CHEZ	4.51	28.7
(m) Tryptophan synthase α -chain	5.30	28.7
(n) Outer-membrane protein A (OmpA)	5.34	28.6
(v) Deoxypyridine-5'-triphosphate nucleotidohydrolase	5.03	16.1
(w) –	4.24	12.2
(x) –	8.27	14.5
(y) –	5.46	10.3
(z) –	6.71	10.4
Protein synthesis is turned off in the presence of Hfq (open arrowheads in Fig. 3)		
(d) –	6.15	44.3
(o) –	4.38	26.5
(p) –	4.82	26.9
(s) –	5.50	20.2
(t) –	6.00	17.4
(u) –	6.28	16.8

in the expression of RpoS-independent genes, we compared the two-dimensional gel electrophoretic patterns of total-cellular-protein synthesis in IM1101(pESE102) and IM1101(pESP82) with that of the RpoS[−] Hfq[−] control strain IM1101(pUC18). As mentioned above, although the protein levels of Hfq_{Ec} and Hfq_{Pa} seem to vary in strains IM1101(pESE102) and IM1101(pESP82) no gross difference was observed in their protein profile. (Fig. 3b–c). This again showed that Hfq_{Pa} can functionally replace Hfq_{Ec} and that the C-terminal end of Hfq_{Ec} is not necessary for its function. When compared to the RpoS[−] Hfq[−] control strain, the synthesis of at least five proteins was found to be enhanced in the presence of Hfq (indicated by solid arrows, Fig. 3a–c), whereas Hfq appears to be essential for the synthesis of four proteins (marked by open arrows, Fig. 3a–c). In

contrast, the synthesis of at least six proteins was found to be turned off in the presence of Hfq (indicated by open arrowheads, Fig. 3a–c) and the synthesis of 11 proteins was decreased in the presence of Hfq (indicated by solid arrowheads in Fig. 3a–c). In accordance with a recent study (Vytvytska *et al.*, 2000), OmpA was found among these 11 proteins (Table 2; protein n). Using the *E. coli* Swiss-2D PAGE, we have attempted to identify the proteins whose synthesis was affected by Hfq. Among the 26 proteins, 11 proteins were tentatively assigned (Table 2, a–z).

Suh *et al.* (1999) have reported that RpoS mediates the resistance of *P. aeruginosa* to heat shock, H₂O₂ and osmotic stress. Additionally, RpoS affects the accumulation of exotoxin A, pyoverdine and alginate, which

seem to be important for *P. aeruginosa* to survive adverse conditions during infection. Given that Hfq_{Pa} affected the expression of *E. coli* *rpoS* (see Fig. 2) in a positive manner it remains to be seen whether it has the same role in *P. aeruginosa*. In addition, studies are currently under way to test whether Hfq_{Pa} directly affects the expression of *P. aeruginosa* genes, as was indicated in this study for a number of *E. coli* genes.

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