The *hfq* gene is required for stress resistance and full virulence of *Burkholderia cepacia* to the nematode *Caenorhabditis elegans*

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**INTRODUCTION**

The *Burkholderia cepacia* complex (Bcc) emerged as problematic opportunistic pathogens to cystic fibrosis (CF) patients. Although several virulence factors have been identified in Bcc, the knowledge of their relative contribution to Bcc pathogenicity remains scarce. In this work, we describe the identification and characterization of a *B. cepacia* IST408 mutant containing a disruption in the *hfq* gene. In other bacteria, Hfq is a global regulator of metabolism, acting as an RNA chaperone involved in the riboregulation of target mRNAs by small regulatory non-coding RNAs (sRNAs). The *B. cepacia* Hfq protein was overproduced as a histidine-tagged derivative, and we show evidence that the protein forms hexamers and binds sRNAs. When provided in trans, the *B. cepacia* IST408 *hfq* gene complemented the *Escherichia coli* *hfq* mutant strain GS081. Our results also show that the *B. cepacia hfq* mutant is more susceptible to stress conditions mimicking those faced by Bcc bacteria when infecting the CF host. In addition, the *B. cepacia hfq* mutant and two *hfq* mutants derived from *B. dolosa* and *B. ambifaria* clinical isolates also exhibited a reduced ability to colonize and kill the nematode *Caenorhabditis elegans*, used as an infection model. These data, together with the conservation of Hfq orthologues among Bcc, strongly suggest that Hfq plays a major role in the survival of Bcc under stress conditions, contributing to the success of Bcc as CF pathogens.

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**Abbreviations:** Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; EPS, exopolysaccharide; IMG, Integrated Microbial Genomes; LB, Lennox broth; sRNAs, small regulatory non-coding RNAs; TB, Terrific broth.

The GenBank/EMBL/DDBJ accession number for the *B. cepacia* IST408 *hfq* sequence is EU760353.

Mahenthiralingam et al., 2005). A remarkable exception to their predominance is the infection by *B. cepacia* of 85% of the Portuguese CF patients who attended the major Portuguese CF Centre during the period between 2003 and 2006 (Cunha et al., 2007). Furthermore, strains of the *B. cepacia* species have also been associated with increased deterioration of lung function and poor clinical outcome, including the cepacia syndrome (Cunha et al., 2007). Several virulence factors have been identified in Bcc (Mahenthiralingam et al., 2005), although information on their relative contribution to the overall pathogenicity remains unclear.

In this work, we describe the isolation and characterization of a mutant from *B. cepacia* IST408, first identified due to its reduced ability to produce the exopolysaccharide (EPS) cepacian. Nucleotide sequence analysis revealed that this mutant carries an insertion in the promoter region of a gene similar to the *Escherichia coli* *hfq* gene. In *E. coli*, *hfq* encodes a 102 aa protein that was first identified due to its requirement for the replication of the RNA plus-strand of bacteriophage Qβ (Franze de Fernandez et al., 1968). The *E. coli* *hfq* mutant exhibits a pleiotropic phenotype, including decreased growth rate and increased sensitivity to UV light, mutagens and oxidants (Tsui et al., 1994).
Moreover, the synthesis of more than 50 proteins was affected in the *E. coli* hfq mutant, partly due to the requirement of Hfq for the efficient translation of the rpoS gene encoding the σ^5^ sigma factor, which is expressed under a variety of stress conditions and in the stationary phase of growth (Muffler et al., 1997). The *E. coli* Hfq protein is a global regulator of the bacterial metabolism, acting as an RNA chaperone; it is involved in the riboregulation of target mRNAs by small regulatory non-coding RNAs (sRNAs), facilitating their interaction with target mRNAs (Valentin-Hansen et al., 2004). Hfq proteins have also been identified as important virulence factors in several Gram-negative bacteria, including *Yersinia enterolitica, Salmonella typhimurium, Pseudomonas aeruginosa, Brucella abortus* and *Neisseria meningitidis*, among others (Fantappie et al., 2009; Nakao et al., 1995; Robertson & Roop, 1999; Sittka et al., 2007; Sonneleitner et al., 2003). Here, we report the identification and cloning of the *B. cepacia* hfq gene and show results indicating the ability of the encoded protein to form hexamers and to bind RNA molecules. Results on the requirement of a functional hfq for resistance to stress conditions and survival in the nematode *Caenorhabditis elegans* are also presented and discussed.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** Bacterial strains and plasmids used in this study are described in Table 1. The *B. cepacia* IST408-SS7 mutant was obtained by random mutagenesis of *B. cepacia* IST408 using the plasposon pTnModOKm (Dennis & Zylstra, 1998), using the methods described by Moreira et al. (2003), and initially selected due to its EPS-defective phenotype. Bcc and *E. coli* strains were maintained, respectively, in *Pseudomonas* isolation agar (PA; Beckton Dickinson) plates or in Lennox broth (LB; Sigma) with 2% (w/v) agar (IberaGen). Unless otherwise stated, liquid cultures were in LB at 37°C with orbital agitation (250 r.p.m.). Bacterial growth was followed by measuring the optical density of the cultures at 640 nm. When appropriate, antibiotics were used at the following concentrations (µg ml^-1^): for *E. coli*, ampicillin (Ap) 150; chloramphenicol (Cm) 25; tetracycline (Tet) 10; trimethoprim (Tp) 200; for *B. cepacia* IST408 and *B. ambifaria* CEP0996, Tp 20; for *B. dolosa* AU0158, Tp 800.

**DNA manipulation techniques.** Total DNA from Bcc strains was extracted from cells harvested from liquid cultures grown overnight, using the Dneasy blood & tissue kit (Qiagen), following the manufacturer’s instructions. Plasmid DNA isolation and purification, DNA amplification and restriction, and agarose gel electrophoresis were performed using standard procedures (Sambrook & Russell, 2001). Plasmid DNA was introduced in *E. coli* and Bcc strains by electroporation as described previously (Moreira et al., 2003).

**Construction of hfq insertion mutants from *B. cepacia* IST408, *B. dolosa* AU0158 and *B. ambifaria* CEP0996.** Primers P3 (5’-TTTGGATCCGGGTGGTTGCAG-3’) and P6 (5’-TTTGGTACC-GACTCGAGGAAGA-3’) containing the BamHI and KpnI restriction sites, respectively, at their 5’ ends, were designed to amplify the *B. cepacia* IST408 2765 bp segment containing the hfg gene and the flanking regions. The amplified fragment was ligated into the BamHI/KpnI sites of pDrive, creating pSAS1 (Table 1). The 986 bp Xho fragment from pUC-Tp (Sokol et al., 1999) containing the Tp cassette was ligated to the single HindIII restriction site within the hfg gene carried on pSAS1, creating pSAS2 (Table 1). This plasmid was introduced into *B. cepacia* IST408, *B. dolosa* AU0158 or *B. ambifaria* CEP0996 by electroporation (Ferreira et al., 2007). Double cross-over recombination resulting in the insertional inactivation of the hfg gene in these Bcc strains was confirmed by PCR using primers P1 (5’-TTTCTCAGACGCGGAGGC-3’) and P2 (5’-AAAGGATCC-AAGGACAAA-3’).

**RT-PCR experiments.** Total RNA was extracted from cells of exponentially growing cultures of the wild-type *B. cepacia* strain IST408 or the hfg mutant *B. cepacia* IST408-SS7 using the SV total RNA isolation system (Promega) followed by DNase I treatment, according to the manufacturer’s instructions. The concentration and purity of extracted RNA samples was determined using a Nanodrop ND-1000 UV/visible spectrophotometer (Nanodrop Technologies).

The RT reaction was carried out using 1 µg total RNA and the superscript one-step RT-PCR kit with platinum Taq (Invitrogen). Control experiments, using either total DNA or total RNA as template in the absence of reverse transcriptase, were also performed. The primer pairs P1 and P2, P3 and P4 (5’-CGGATCCGATTTCGGCAA-3’), P5 (5’-CGGTACCGTGGTTTCGAGC-3’) and P6, were used to investigate transcription from hfg, hflX and/or engA, respectively. Primers P2 and P3 were used to investigate hfg–hflX co-transcription.

**RNA extraction, purification and labelling.** mRNA and sRNA fractions were obtained from *B. cepacia* IST408 cells at the late exponential phase of growth, using the mirVana miRNA isolation kit (Ambion) according to the manufacturer’s instructions. RNA quality was assessed using an Agilent Bioanalyser ACF (Agilent Technologies) and by visual inspection of the RNA after electrophoresis in 8 % urea polyacrylamide gels.

sRNAs were purified from bands excised from the gel, squashed using an RNase-free pipette tip, and eluted overnight at 4°C in TE buffer (pH 8.0). Eluted sRNAs were ethanol-precipitated and resuspended in high-purity nuclease-free water. sRNAs were dephosphorylated using 1 U calf intestinal phosphatase (New England Biolabs) following the manufacturer’s instructions. sRNAs were end-labelled using the T4 polynucleotide kinase (Gibco-BRL) with fluorescein-labelled nucleotide mix (GE Healthcare). Labelled sRNAs were ethanol-precipitated and resuspended in 25 µl RNA binding buffer [containing 20 mM Tris/HC1 (pH 8.0), 20 mM KCl, 1 mM MgCl2, 10 mM sodium phosphate buffer (pH 8.0) and 1 mM DTT].

Hfq cloning, overproduction and purification. The *B. cepacia* IST408 hfg gene was PCR amplified from chromosomal DNA using primers Hfq_UP_E (5’-GAATTCATGAGCAACAAAAGG-3’) and HF_Low_E (5’-TTTGGGATCCTCGAGGAGGCAA-3’), containing EcoRI and Xhol restriction sites, respectively. After hydrolysis, the gene was ligated into EcoRI/Xhol-digested pET23a+, creating pCGR4 (Table 1). *E. coli* BL21 (DE3) harbouring pCGR4 was grown at 30°C in 100 ml Terrific broth (TB) medium (Sambrook & Russell, 2001) with 150 µg ampicillin ml^-1^, until an OD_{600} of 0.1 was reached. Cells were harvested by centrifugation, washed once with 1 × PBS buffer (pH 7.2) and used to inoculate 500 ml TB medium with 150 µg ampicillin ml^-1^.

The culture was incubated at 30°C until an OD_{600} of 0.5 was reached, then IPTG (0.4 mM final concentration) was added. After 3 h incubation, cells were harvested by centrifugation. Cell lysis and protein purification were performed as described previously (Sousa et al., 2007), using an Ni-NTA column (Amersham). Fractions containing Hfq were dialysed overnight at 4°C against storage buffer [20 mM Tris/HC1 (pH 8.0), 100 mM KCl, 5 mM MgCl2, 50 mM Tris base, 0.01% (w/v) Tween 20 and 1 mM DTT] using a 10 kDa cutoff Slide-A-Lyser (Pierce). Protein concentration was estimated using the Bradford method (Bradford, 1976). Protein purity was confirmed by visual inspection of 15% SDS-PAGE gels run.
(w/v) polyacrylamide gels, after electrophoresis and Coomassie blue staining.

Electrophoretic mobility shift assays (EMSA). Purified His-tagged Hfq was incubated at 30 °C for 15 min with 16 nmol fluorescent-labelled sRNA in 25 µl RNA binding buffer. Control mixtures were also prepared, lacking the purified His-tagged Hfq protein. To minimize non-specific binding, non-labelled total RNA, extracted as described above, was added in excess to each reaction. One microlitre of loading buffer (Ambion) was added to each sample and the resulting mixtures were loaded on a 10% (w/v) polyacrylamide gel in 0.5x TBE buffer (Sambrook and Russell, 2001) containing 0.5% (w/v) glycerol. After electrophoresis, performed at 4 °C and 350 V, the gel was scanned using a Typhoon 8600 variable mode imager, using fluorescein emission, blue filter, 600 V excitation and 100 µm resolution (Amersham Biosciences Europe).

In vitro cross-linking of B. cepacia His-tagged Hfq. Cross-linking experiments with purified His-tagged Hfq were performed by formaldehyde treatment, based on the methods described by Jackson (1999). Briefly, reaction mixtures containing 50–100 µg purified His-tagged Hfq in 100 µl 20 mM HEPES buffer (pH 7.5) were treated with 5 µl of a 2.3% (w/v) freshly prepared formaldehyde solution for 2–5 min at 37 °C. The reaction was stopped by the addition of 10 µl 1 M Tris/HCl (pH 8.0). These reaction mixtures containing the cross-linked proteins were mixed with an equal volume of Laemmli sample buffer (Sambrook & Russell, 2001), to which 2 µl 0.1% (w/v) bromophenol blue was added. Electrophoresis was carried out using 15% (w/v) SDS-polyacrylamide gels.

The Hfq–sRNA complexes, obtained as described above for the EMSAs, were treated by UV irradiation for 5 min on ice; 2.5 U RNase A was added (Citomed) and the mixture was incubated for 1 h at 37 °C. The His-tagged Hfq–RNA complexes were analysed by native PAGE.

Nematode killing assays and bacterial colonization. Nematode slow-killing assays were performed and bacterial colonization of the nematodes’ digestive tract was measured for B. cepacia strains IST408, the hfg mutant strain SJ1 and SJ1 harbouring plasmid pSAS3, based on the methods of Moy et al. (2006) and as described previously (Sousa et al., 2008). The C. elegans mutant strain DH26 was used since this strain has a temperature-sensitive mutation, rendering worms sterile at 25 °C, allowing the score of worms without the interference of progeny (Roberts & Ward, 1982). Slow-killing experiments were carried out at least five times using five plates per experiment. E. coli OP50 was used as a negative control. Nematode killing assays and bacterial colonization were also performed for B.
**RESULTS**

**Cloning and sequence analysis of the** *B. cepacia* **IST408 hfq gene**

The *B. cepacia* IST408-SS7 mutant was identified due to its EPS-defective phenotype during the screening of a mutant library; it is derived from the high EPS producer *B. cepacia* IST408 (Richau et al., 2000). The presence of a single plasposon insertion within the genome of the *B. cepacia* IST408-SS7 mutant was confirmed by Southern blot using EcoRI-digested chromosomal DNA and, as a probe, the 1 kb DNA fragment containing the kanamycin resistance cassette, obtained by Clal restriction of pTiModOKm (data not shown). In order to identify the interrupted gene, total DNA from *B. cepacia* IST408-SS7 was extracted, digested with EcoRI, self-ligated and used to transform *E. coli* DH5α. The DNA insert was sequenced with primers 5’ KMR and ORIR-1 (Moreira et al., 2003). Analysis of the nucleotide sequence revealed that the plasposon was inserted 132 bp upstream of the ATG codon of a putative gene encoding a protein 81% identical to the *E. coli* Hfq (Fig. 1a, c). The *B. cepacia* IST408 hfq-like gene was predicted to encode a 79 aa protein of 8.85 kDa with a pl of 8.1. The nucleotide sequence of *B. cepacia* IST408 hfq was deposited in GenBank (accession no. EU760353). The *B. cepacia* IST408 Hfq secondary structure was predicted to contain an N-terminal z-helix followed by five β-strands, in an arrangement quite similar to the structure of *E. coli* Hfq (Fig. 1a, b), determined at a 2.15 Å resolution (Sauter et al., 2003) (PDB ID: 1HK9). Two conserved Sm motifs were predicted. The Sm1 motif encompasses the first three β strands and the Sm2 motif encompasses the fourth and fifth β strands (Fig. 1a). Sm motifs are found in Sm and Sm-like eukaryotic and archaeal proteins that form heteroheptamers and participate in RNA processing reactions (Pannone & Wolin, 2000). The *B. cepacia* Hfq contains the conserved amino acid residues Gln9, Phe40, Lys57 and His58, which have been demonstrated to be involved in RNA binding by *E. coli* Hfq (Fig. 1a), and Gly30, which allows the bending of β strand 2 (Sauter et al., 2003). Loops 3 and 5 are present in the central nucleotide binding pocket, as indicated in Fig. 1a (Sauter et al., 2003). In the *E. coli* Hfq, the C-terminal extension after the β5 strand is composed of 38 aa, mainly hydrophilic, with no predicted 2D structure (Sauter et al., 2003). The *B. cepacia* IST408 Hfq is 24 aa shorter (Fig. 1a).

**The hfq locus is highly conserved among Bcc species**

A gene 99% identical to the *B. cepacia* IST408 hfq-like gene was found within the genome sequence of *B. cenocepacia* J2315, spanning nt 2074649–2074888 of chromosome 1 (BCAL 1879). Genes homologous to the *E. coli* hfIC, hfIK, hfIX and engA are also located in this locus (Fig. 1c). The putative genes hfIC, hfIK and hfIX and their order of appearance in this *B. cenocepacia* J2315 locus are similar to the corresponding homologues in *E. coli* (Tsui et al., 1994). In *E. coli*, HfIX is a GTPase that binds to the ribosome 50S subunit, while hfIK and hfIC encode membrane-associated proteases (Dutta et al., 2009), although the precise physiological roles of these proteins remain largely unknown. The engA gene upstream of *B. cenocepacia* J2315 hfq is not found in the *E. coli* hfq locus. In *E. coli*, the genes immediately upstream of hfq are miaA–mutl–amiB (Tsui et al., 1994). EngA is an essential GTPase composed of dolosa strains AU0158 and its hfq derivative mutant, and for *B. ambifaria* CEP0996 and its hfq derivative mutant.

**Susceptibility to high temperature, salt, ethanol and methyl viologen.** Cultures of *B. cepacia* strains IST408, SJ1 or SJ1(pSAS3), and *E. coli* strains MC4100, GS081 or GS081(pCRG5) were grown for 3 h at 37 °C with orbital agitation in LB liquid media with appropriate antibiotics. Cells were then harvested by centrifugation, washed once with sterile saline (0.9% w/v) and resuspended in sterile water to an OD600 of 1.0, which corresponded to (2.0 ± 1) × 108 c.f.u. ml⁻¹ for *B. cepacia* strains and (8.0 ± 2) × 107 c.f.u. ml⁻¹ for *E. coli* strains. Serial dilutions were prepared and 10 μl of each dilution was spotted on to the surface of LB plates, supplemented with 5% (v/v) ethanol, 3.5% (v/v) NaCl or 150 μM methyl viologen in the case of *Burkholderia* strains, or 3% ethanol, 2.5% NaCl or 50 μM methyl viologen in the case of *E. coli* strains. Spots were allowed to dry and plates were incubated at 37 or 42 °C for 48 h. Results shown are representative of at least three independent experiments.

**Survival to long-term nutrient deprivation.** Adequate volumes from 25 ml liquid cultures of *B. cepacia* strains IST408 and SJ1 contained in 100 ml Erlenmeyer flasks and grown overnight in LB at 37 °C with agitation, were harvested by centrifugation, washed twice with sterile saline solution and resuspended in 100 ml M9 minimal medium (Sambrook & Russell, 2001) contained in 250 ml Erlenmeyer flasks, supplemented with 0.002% (w/v) each of l-leucine, l-isoleucine, l-valine, l-methionine, l-arginine, tryptophan, l-phenylalanine and l-histidine, and 0.1% (w/v) glucose, to a final OD600 of 1.0 [(2.0 ± 0.1) × 109 c.f.u. ml⁻¹]. Cultures were incubated for 30 days at 37 °C. The number of surviving bacteria was assessed by quantification of c.f.u. in PIA plates after 48 h incubation at 37 °C. Results shown are representative of four independent experiments and are expressed as the percentage of the initial c.f.u. value.

**Swimming motility assays.** Motility assays were performed based on the methods of Bernier & Sokol (2005). Briefly, 1 ml exponentially growing *B. cepacia* strains IST408, SJ1 or SJ1(pSAS3) cultures with an OD600 of 2.0 [containing (2.30 ± 0.05) × 109 c.f.u. ml⁻¹] was spotted on the surface of a Swim agar plate containing Nutrient Broth (Becton Dickinson), 0.25% (w/v) agar and 1% (w/v) l(+)-arabinose. The plates were allowed to dry for 1 h at room temperature and then incubated for 16–22 h at 30 °C. Swimming zone diameters were measured and imaged in a Universal Hood II (Bio-Rad). Results are the mean values of at least three independent experiments.

**Growth and quantification of EPS production.** EPS production was quantified by calculating the dry weight of the ethanol-precipitated polysaccharide present in 100 ml cell-free culture samples of *B. cepacia* strains IST408 and SJ1, cultivated in S liquid medium, for 72 h at 30 °C, with orbital agitation (250 r.p.m.), as described previously (Richau et al., 2000). EPS production was quantified as EPS weight (g) per litre of growth medium and values given are the mean values of at least three independent determinations.
**Fig. 1.** *B. cepacia* IST408 Hfq amino acid sequence, predicted structure and genetic and transcriptional organization of the *hfq* chromosomal locus. (a) Amino acid sequence alignment of the *B. cepacia* IST408 Hfq protein with the indicated bacterial Hfq proteins. Loops 3 (L3) and 5 (L5) are boxed in grey. Asterisks (*) above the sequence indicate conserved residues involved in RNA binding by *E. coli* Hfq. An asterisk below the sequence indicates conserved amino acid residues; dots or double dots indicate semi-conserved or conserved substitutions, respectively. (b) Structural model of *B. cepacia* IST408 and *E. coli* K-12 (PDB ID: 1HK9) Hfq proteins. Graphics were generated using the RasWin Molecular Graphics (Windows Version 2.7.3.1). (c) Schematic representation of *B. cenocepacia* J2315 *hfq* locus. The positions of primers used for RT-PCR are indicated by arrows. The nucleotide position in chromosome 1 is indicated below the diagram. The filled triangle indicates the plasposon insertion 132 bp upstream of the *hfq* ATG codon in the *B. cepacia* IST408-SS7 mutant. (d) Agarose gel showing the results from RT-PCR with the indicated primers. M, 1 kb plus DNA ladder (Invitrogen); IST408, *B. cepacia* IST408 RNA sample; SJ1, *B. cepacia* SJ1 RNA sample; C, *B. cepacia* IST408 DNA.
of two GTP-binding domains arranged in tandem which binds to the ribosome subunits 50S, 70S and 30S, and participates in ribosome biogenesis (Tomar et al., 2009).

In order to gain clues on the transcriptional organization of the identified locus in \textit{B. cepacia} IST408, we performed RT assays (Fig. 1c). No DNA amplification could be detected in RT experiments performed using total RNA from \textit{B. cepacia} IST408-SS7 and primers P1 and P2 (Fig. 1d). Similar experiments using total RNA from the wild-type strain \textit{B. cepacia} IST408 led to the amplification of a 258 bp DNA fragment corresponding to \textit{hfq} (Fig. 1d). These results indicate that the insertion of the plasposon 132 bp upstream of the ATG start codon abolished \textit{hfq} transcription in \textit{B. cepacia} IST408 (Fig. 1c). Furthermore, in \textit{E. coli}, \textit{hfq} is part of the superoperon \textit{amiB}–\textit{mutL}–\textit{miaA}–\textit{hfq}–\textit{hflX}–\textit{hflK}–\textit{hflC} with a complex transcriptional organization (Tsui et al., 1994). Primers P3 to P6 were used to investigate whether \textit{hfq} is co-transcribed with \textit{hflX} or with \textit{engA} in \textit{B. cepacia} IST408 and if the plasposon insertion also affected the transcription of the genes \textit{hflX} and \textit{engA} (Fig. 1d). No amplification products were obtained in RT experiments with primers P2 and P3, indicating that in \textit{B. cepacia}, \textit{hfq} is not co-transcribed with \textit{hflX} (Fig. 1d). RT experiments with primers P1 and P6 did not yield any amplification fragment, indicating that \textit{hfq} is not co-transcribed with \textit{engA} (data not shown). Furthermore, results obtained with the primer pairs P3 plus P4, and P5 plus P6, also indicate that \textit{hflX} and \textit{engA} are both transcribed in the \textit{hfq} mutant strain SI1, even in the absence of \textit{hfq} transcription (Fig. 1d). Together, our RT experiments demonstrate that in \textit{B. cepacia} IST408, the \textit{hfq} gene is not co-transcribed with either of the adjacent \textit{engA} or \textit{hflX} genes.

The presence of \textit{B. cepacia} IST408 \textit{hfq} homologues within the genome sequences of other Bcc strains was investigated bioinformatically, using the complete Bcc genome sequences deposited in the Integrated Microbial Genomes (IMG) database (Markowitz et al., 2008). These analyses revealed that the \textit{hfq} gene sequence is highly conserved among Bcc members, with an identity of at least 95% at the amino acid level (data not shown). In addition, the genetic organization \textit{engA}–\textit{hfq}–\textit{hflX}–\textit{hflK}–\textit{hflC} was also conserved in all the Bcc genome sequences in IMG.

\textbf{B. cepacia IST408 Hfq binds to sRNAs}

To investigate whether the Hfq protein from \textit{B. cepacia} IST408 has the ability to bind sRNAs, the protein was overproduced as a hexa-histidine derivative. sRNAs were isolated from \textit{B. cepacia} IST408 with a commercial kit, labelled with fluorescein and incubated with 0, 25, 50, 100, 250 or 500 nmol purified His-tagged Hfq. The \textit{B. cepacia} IST408 His-tagged Hfq formed complexes with sRNAs, as shown by the retardation profiles of the fluorescein-labelled sRNAs when previously incubated with 25, 50, 100 or 500 nmol Hfq (Fig. 2a). In experiments carried out using 500 nmol Hfq, the Hfq–sRNA complex migrated significantly less distance than the other Hfq concentrations. We have no explanation for the observed relatively higher intensity of the Hfq–sRNA complex formed when using 500 nmol Hfq. In order to investigate the possible formation of Hfq multimers complexed with sRNAs, we performed UV cross-linking experiments of the purified protein after incubation with sRNAs and prior to application on the native gel. The UV cross-linked protein complexed with sRNAs was further treated with RNase and, after electrophoresis, migrated with a retardation pattern identical to the untreated protein (Fig. 2a). Based on these results which suggest the formation of Hfq multimers, we conducted \textit{in vitro} cross-linking assays using the formaldehyde method described by Jackson (1999), followed by SDS-PAGE analysis. Two distinct bands were observed, one with an apparent molecular mass of approximately 14 kDa and corresponding to the Hfq monomer, and the other form with an apparent molecular mass consistent with the formation of Hfq hexamers (Fig. 2b). Together, our results indicate that the \textit{B. cepacia} Hfq is able to form hexamers and to bind sRNAs.

\textbf{B. cepacia IST408 hfq complements an \textit{E. coli} hfq mutation}

Since the \textit{B. cepacia} IST408 Hfq protein is 23 aa shorter than the \textit{E. coli} Hfq protein, we decided to investigate whether \textit{B. cepacia} hfq complements the \textit{E. coli} hfq mutant strain GS081. The \textit{B. cepacia} IST408 \textit{hfq} gene was PCR-amplified with primers HQQcomp-UP (5’-AAGAATTCATGAGCAACAAAG-3’) and HQQcomp-Low (5’-TCATGAGCAACAAAG-3’), and the PCR product was subcloned into the plasmid pET23d-Low, from which the \textit{hfq} gene was expressed as a hexahistidine fusion protein.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{The \textit{B. cepacia} IST408 Hfq protein binds sRNAs and forms hexamers. (a) A clear retardation pattern is observed with increasing amounts of Hfq. At the saturation ratio (30:1 of Hfq:sRNA), an enhanced retardation pattern was observed. After UV cross-linking and hydrolysis of the unbound and partially bound RNA, the same retardation was observed. (b) SDS-PAGE analysis of \textit{in vitro} cross-linking of Hfq, revealing the monomeric and hexameric forms.}
\end{figure}
The amplified 255 bp DNA fragment was digested with EcoRI and XbaI, ligated into the pPHU231 low-copy-number cloning vector (Hübner et al., 1991), yielding plasmid pCGR5 which was introduced into E. coli GS081. pPHU231 was derived from the low-copy-number pRK290 which allows constitutive expression of hfq. In E. coli, Hfq levels are maintained at a characteristic level in cells at different growth phases (Ali Azam et al., 1999) and pPHU231 was used since it allows the maintenance of controlled amounts of Hfq sufficient to complement the mutation. E. coli GS081 harbouring the cloning vector pPHU231 was used as a control. Aliquots of 10 μl of serially diluted suspensions were spotted onto the surface of LB and incubated for 48 h at 42 °C or LB supplemented with 3 % ethanol, 2.5 % NaCl or 50 μM methyl viologen and incubated for 48 h at 37 °C. As shown in Fig. 3a, the B. cepacia IST408 hfq gene restored the ability of E. coli hfq mutant GS081 to survive exposure to the selected stresses, clearly indicating that the B. cepacia IST408 hfq gene is functional in E. coli. The introduction of pPHU231 into E. coli GS081 led to results similar to those observed for the hfq mutant strain E. coli GS081 (data not shown).

**hfp plays a role in stress tolerance in B. cepacia**

We have compared the resistance of the B. cepacia hfp mutant SJ1 and the wild-type to several environmental stresses, mimicked by the addition of ethanol, NaCl or methyl viologen to growth medium, or by growing cultures at 42 °C. The hfp mutant grew poorly at 42 °C compared with the wild-type strain (Fig. 3b). The B. cepacia hfp mutant also exhibited an increased susceptibility to oxidative and osmotic stresses (Fig. 3b). Results also indicate that providing the hfp gene in trans partially restored the wild-type phenotypes (Fig. 3b). Higher concentrations of the stressing agents (ethanol, NaCl or methyl viologen) had to be used when assaying stress resistance of Bcc strains compared with those used for E. coli strains (Fig. 3), suggesting that Bcc strains are less sensitive to the studied stressing agents.

**hfp is required for B. cepacia IST408 survival following prolonged nutrient starvation and exposure to acidic conditions**

The survival of the B. cepacia IST408 wild-type strain and of the hfp mutant SJ1 to prolonged periods of nutrient starvation was compared by following each strain’s growth (assessed by measuring c.f.u.) over a period of 30 days at 37 °C in M9 minimal medium. Our results clearly demonstrate that hfp plays an important role in survival following prolonged periods of starvation (Fig. 4a). We have also compared the resistance of the wild-type and the mutant strain to acidic conditions by spot-inoculating serially diluted cell suspensions in LB medium buffered at pH 5.0 with 50 mM phosphate buffer. For the hfp mutant B. cepacia SJ1, growth was only observed in the first two spots corresponding to a total of $8 \times 10^6$ and $8 \times 10^5$ c.f.u. inoculated. These results clearly indicate that a functional hfp is required for B. cepacia survival under acidic conditions (Fig. 4b).

**hfp is required for full virulence of B. cepacia IST408 and other Bcc strains to C. elegans**

In bacterial pathogens, hfp deletion has been reported to affect the bacterial susceptibility to host defence mechan-

![Fig. 3.](image)
isms, with the mutants exhibiting an attenuated phenotype in infection models (Ding et al., 2004; McNealy et al., 2005; Sittka et al., 2007; Sonnleitner et al., 2003). The role played by hfq on the virulence of B. cepacia IST408 was assessed using the nematode C. elegans, successfully used to assess the virulence traits and mechanisms of bacterial pathogens such as P. aeruginosa (Mahajan-Miklos et al., 1999) and Bcc bacteria (Cardona et al., 2005; Huber et al., 2004; Sousa et al., 2008). The bacteria surviving within the nematodes' digestive tract were also quantified by measuring c.f.u. The B. cepacia IST408 hfq mutant SJ1 exhibited an impaired ability to kill nematodes (Fig. 5a). Consistently, lower numbers of total c.f.u. were detected inside the digestive tract of worms infected with the hfq mutant strain compared with worms infected with the wild-type strain (Fig. 5b). In addition, worms infected with the wild-type strain exhibited a sicker appearance when compared with those affected by the hfq mutant (Fig. 5c).

In order to gain further clues about the role of hfq on the pathogenicity of other Bcc species, we have prepared hfq mutants from the CF isolates B. dolosa AU0158 and B. ambifaria CEP0996. Although several attempts to generate hfq mutants from the CF isolates B. cenocepacia J2315 and B. multivorans LMG16660 have been carried out, we were not able to isolate hfq mutants from these strains. B. dolosa and B. ambifaria hfq mutants also exhibited a decreased ability to kill C. elegans and to colonize the digestive tract of the worms (Fig. 6). The worms infected by wild-type strains of B. dolosa and B. ambifaria became thinner and exhibited a sicker appearance when compared with those infected by the respective hfq mutants (Fig. 6c). Together, these results indicate a role for hfq in the pathogenicity of Bcc bacteria.

**hfq plays a role in EPS biosynthesis and motility in B. cepacia IST408**

The possible role played by hfq on EPS biosynthesis was assessed by comparing the amount of EPS produced by the wild-type strain B. cepacia IST408 and the hfq mutant strain SJ1, in S liquid medium. The B. cepacia IST408 hfq mutant produced about half of the EPS produced by the wild-type strain (Fig. 7a), suggesting that hfq plays a role in the regulation of EPS biosynthesis in B. cepacia and, most probably, in other Bcc species.

In the pathogens S. typhimurium and P. aeruginosa, disruption of hfq partially impairs the bacterial cell motility (Sittka et al., 2007; Sonnleitner et al., 2003). During the colonization and infection processes of Bcc, motility endows the pathogen with a survival advantage, enhancing bacterial colonization and persistence (Tomich et al., 2002). Decreased migration (swimming) was observed for the B. cepacia hfq mutant compared with the wild-type strain (Fig. 7b). The motility defect was rescued by complementation with pSAS3 (Table 1), which expressed the hfq gene in trans (Fig. 7b). These results suggest that, although Hfq is not absolutely required for B. cepacia motility, it interferes with the motility of B. cepacia.

**A second putative hfq-like gene is present within the B. cepacia IST408 genome**

The remarkable differences observed when comparing the effects of complementation of the hfq mutations in E. coli MC4100 or in B. cepacia IST408 prompted us to search for putative hfq homologues in the available genome sequence of B. cenocepacia J2315. This analysis allowed the identification of BCAL1538, putatively encoding an hfq-like gene. Using primers hfq2up (5'-AAGATCCATGGCC-AATCCCGCAGA-3') and hfq2low (5'-TTGTCGACTT-ACTGGCCGTCCG-3'), designed based on the B. cenocepacia J2315 genome sequence, and total DNA isolated from B. cepacia IST408 as template, a 580 bp amplicon was obtained by PCR (Fig. 8). The presence of an additional gene putatively encoding an Hfq-like protein within the genome of B. cepacia IST408 might explain the less dramatic effects of the insertional inactivation of hfq in B. cepacia IST408 when compared with those observed for E. coli MC4100. Work is in progress on the functional analysis of BCAL 1538, encoding a putative Hfq-like protein.
DISCUSSION

In the present work, we report the identification and characterization of a *B. cepacia* IST408 mutant in which expression of a gene putatively encoding the RNA chaperone Hfq is abolished, as well as the characterization of the ability of the protein to form hexamers *in vitro* and to bind sRNA molecules. The *B. cepacia* IST408 *hfq* mutant was identified based on a strategy that led us to identify the *bce* cluster of genes directing the synthesis of the EPS cepacian (Moreira et al., 2003). Although genes involved in the synthesis of EPS have been described and a few have been functionally characterized (Ferreira et al., 2007; Loutet et al., 2009; Sousa et al., 2007; Videira et al., 2005), the regulatory mechanisms underlying the regulation of EPS biosynthesis in Bcc remain unknown. A reduction of 20% in the yield of the alginate produced was reported for a *P. aeruginosa* *hfq* mutant (Sonnleitner et al., 2003). The *B. cepacia* IST408 *hfq* mutant produced only

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**Fig. 5.** The *hfq* gene is required for *B. cepacia* IST408 virulence to the nematode *C. elegans*. Comparison of the ability of *B. cepacia* IST408 (black bars), *B. cepacia* SJ1 (white bars) and *B. cepacia* SJ1(pSAS3) (shaded bars) to kill the nematode *C. elegans* DH26 (a). Bacterial counts in the nematodes’ intestinal tract (b) after infection for the indicated time, and photographs of randomly chosen nematodes (c) to visualize their physical status (magnification ×40). Error bars in (a) and (b) indicate SD.

**Fig. 6.** The *hfq* gene is required for full virulence of *Bcc* to the nematode *C. elegans*. Comparison of the ability of *B. dolosa* AU0158 (black bars/1), *B. dolosa* SJ1 (white bars/2), *B. ambifaria* CEP0996 (shaded bars/3) and *B. ambifaria* SJ1 (hatched bars/4) to kill *C. elegans* DH26 (a), the bacterial counts in the nematodes’ intestinal tract (b) after infection for the indicated time, and photographs of randomly chosen nematodes (c) to visualize their physical status (magnification ×40). Error bars in (a) and (b) indicate SD.
about half the amounts of EPS produced by the wild-type strain. The molecular mechanisms linking Hfq and the regulation of EPS biosynthesis in *B. cepacia* are presently unknown. However, due to the roles reported for Hfq in other bacteria, we anticipate an indirect role, most probably involving other regulatory proteins.

In *E. coli*, Hfq is a highly abundant protein and mutations in the encoding gene lead to pleiotropic phenotypes, mainly due to the role of Hfq as an RNA chaperone that allows the interaction of sRNAs and their target mRNAs (Brennan & Link, 2007). The encoding gene is located in the *amiB–mut1–miaA–hfq–hfIX–hfIK–hfIC* superoperon with multiple $\sigma^E$- and $\sigma^70$-dependent promoters, originating various polycistronic messengers (Tsui *et al.*, 1994). This genetic arrangement differs from that found in *B. cepacia* IST408, in which *hfq* is located in a locus composed of the genes *engA–hfq–hfIX–hfIK–hfIC*. In addition, our reverse transcription experiments also revealed that the *B. cepacia* *hfq* locus transcriptional organization differs from that reported for *E. coli*, with *hfq* being transcribed as a single monocistronic messenger independently of the adjacent *engA* and *hfIX* genes. The locus where *hfq* locates is highly conserved among Bcc bacteria. Since in Bcc strains the locus is composed of the same genes and arranged in the same order as that described for *B. cepacia* IST408, we hypothesize that in Bcc bacteria, the transcription organization of the *hfq* locus is most probably identical to that reported here for *B. cepacia* IST408.

Even though *B. cepacia* IST408 Hfq is 24 aa shorter at the C-terminal end compared with the *E. coli* Hfq protein, our results clearly indicate that the *B. cepacia* IST408 *hfq* gene is able to complement the *E. coli* *hfq* mutant strain GS081. It is worth noting that results from the phenotypic characterization and complementation experiments were more impressive when compared with those observed for *B. cepacia* IST408. In light of the reported occurrence of genes in multiple copies within Bcc genomes, as is the case, for example, for the bifunctional BceA proteins with phosphomannose isomerase/GDP–mannose pyrophosphorylase activities (Sousa *et al.*, 2007), we performed a bioinformatics search for putative Hfq proteins encoded within the *B. cenocepacia* J2315 genome sequence. This analysis allowed the identification of BCAL1538, putatively encoding an unusual Hfq-like protein composed of 189 aa. Whether this gene is present and functional in *B. cepacia* IST408 is unknown. Nevertheless, our results clearly indicate that if a BCAL1538 homologue is present within the genome of *B. cepacia* IST408, it does not fully

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**Fig. 7.** Mutation of the *hfq* gene affects EPS production and the motility of *B. cepacia* IST408. (a) Growth curves (circles) and EPS production (bars) by *B. cepacia* IST408 (black) and *B. cepacia* SJ1 (white). (b) Bars showing the spread of *B. cepacia* IST408, the *hfq* mutant SJ1 and SJ1(pSAS3) at 30 °C, 16 h (black bars) or 22 h (white bars) after inoculation onto swimming agar plates. Photographs of swimming agar plates (above) were taken after 16 h incubation. Error bars indicate SD.

**Fig. 8.** Ethidium bromide-stained agarose gel showing the amplification product obtained with primers hfq2up and hfq2low, using *B. cepacia* IST408 total DNA as template (lane 1), demonstrating the presence of BCAL1538 within the genome sequence of *B. cepacia* IST408. Lane 2, molecular mass marker.
compensate the mutation in the hfq gene studied in this work. Research to functionally characterize the protein encoded by BCAL1538 is in progress.

The B. cepacia IST408 Hfq protein is able to bind sRNAs, as demonstrated by EMSAs. Since we used sRNAs purified from cells, no information is available on the specific identity and nucleotide sequences of the sRNAs that bind to B. cepacia IST408 Hfq. In E. coli, several sRNAs and their biological roles have been described, such as the OxyS (Zhang et al., 2002), which is part of the OxyR-regulated transcriptional response to oxidative stress, and RyhB, regulated by the Fur global regulator of iron acquisition genes [reviewed by Gottesman (2005)]. Although no experimental data are presently available on sRNAs and their roles in Bcc strains, 213 putative sRNAs were recently identified within the genome of B. cenocepacia J2315 based on the combination of comparative genomics and prediction of their secondary structures (Coenye et al., 2007). Work is in progress to identify sRNAs that specifically bind to Hfq, envisaging the understanding of their roles on the biology and virulence of Bcc bacteria.

The B. cepacia IST408 hfq mutant exhibited increased susceptibility to conditions related to survival under osmotic, oxidative and acidic stress conditions, reduced motility, and impaired pathogenesis to the infection model C. elegans, consistent with phenotypes described for other bacteria, such as the pathogens B. abortus (Robertson & Roop, 1999) and N. meningitidis (Fantappiè et al., 2009). A key factor in the development of disease by N. meningitidis is the ability of the pathogen to colonize the hostile environment of the mucosal epithelium and to multiply within human blood. An N. meningitidis hfq mutant was attenuated in ex vivo and in vivo models of infection (Fantappiè et al., 2009). An hfq mutant of the intracellular pathogen B. abortus failed to replicate within cultured murine macrophages and was rapidly cleared from the spleens and livers of mice (Robertson & Roop, 1999). Remarkably, some aspects of the pathogenesis of Bcc bacteria and survival when infecting CF patients, thus contributing to the success of these bacteria as pathogens in CF.

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