Microbiology (2008), **154**, 3134–3143 DOI 10.1099/mic.0.2008/020222-0

The cyanobacterial homologue of the RNA chaperone Hfq is essential for motility of *Synechocystis* sp. PCC 6803

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Received 2 May 2008
Revised 3 July 2008
Accepted 7 July 2008

The *ssr3341* locus was previously suggested to encode an orthologue of the RNA chaperone Hfq in the cyanobacterium *Synechocystis* sp. strain PCC 6803. Insertional inactivation of this gene resulted in a mutant that was not naturally transformable and exhibited a non-phototactic phenotype compared with the wild-type. The loss of motility was complemented by reintroduction of the wild-type gene, correlated with the re-establishment of type IV pili on the cell surface. Microarray analyses revealed a small set of genes with drastically reduced transcript levels in the knockout mutant compared with the wild-type cells. Among the most strongly affected genes, *slr1667, slr1668, slr2015, slr2016* and *slr2018* stood out, as they belong to two operons that had previously been shown to be involved in motility, controlled by the cAMP receptor protein SYCRP1. This suggests a link between cAMP signalling, motility and possibly the involvement of RNA-based regulation. This is believed to be the first report demonstrating a functional role of an Hfq orthologue in cyanobacteria, establishing a new factor in the control of motility.

INTRODUCTION

Cyanobacteria constitute a diverse group of photoautotrophic bacteria that perform oxygenic photosynthesis and are present in almost any environment on this planet, such as freshwater, oceans, the surface of rocks, desert soil or the polar regions. Analogous to enterobacteria, in which all responses to environmentally relevant stress conditions have been suggested to include at least one small regulatory RNA (sRNA) as part of the regulon (Gottesman, 2005), cyanobacteria are likely to possess sophisticated riboregulatory mechanisms. Up to now, there have only been a few studies that have identified or functionally characterized sRNAs in cyanobacteria. Essentially, these investigations have been focused on the unicellular marine *Prochlorococcus* group (Axmann *et al.*, 2005; Steglich *et al.*, 2008), the unicellular freshwater cyanobacterium *Synechocystis* sp. PCC 6803 (Dühring *et al.*, 2006) and the filamentous strain *Anabaena* sp. PCC 7120 (Hernandez *et al.*, 2005). In the latter two cases, the involvement of an antisense RNA in iron homeostasis was demonstrated. The *Synechocystis* sp. PCC 6803 antisense RNA IsrR proved to negatively control the expression of the accessory photosynthesis protein IsiA (iron-stress-induced protein A), which dramatically accumulates upon several environmental challenges including iron depletion (reviewed by Singh & Sherman, 2007).

In *Escherichia coli* and closely related bacteria, the functional efficiency and stability of a considerable number of sRNAs is dependent on, or decisively enhanced by, the RNA-binding protein Hfq (Sledjeski *et al.*, 2001; Møller *et al.*, 2002; Zhang *et al.*, 2002; Geissmann & Touati, 2004; Gottesman, 2004; Udekwu *et al.*, 2005; Kawamoto *et al.*, 2006; Urban & Vogel, 2008; Pfeiffer *et al.*, 2007; Večerek *et al.*, 2007). The homohexameric Hfq protein is structurally and functionally similar to eukaryotic Sm proteins (Valentin-Hansen *et al.*, 2004). Its regulatory activity for *E. coli* has been postulated to faciliate the coupled degradation of sRNA–mRNA-duplexes in concert with the major

Abbreviations: Cm, chloramphenicol; CRP, cAMP receptor protein; Km, kanamycin; sRNA, small regulatory RNA; WT, wild-type.

The microarray data discussed in this publication have been deposited in NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE10708.

A supplementary figure showing alignment of Hfq from *Synechocystis* sp. 6803 with other bacteria, and a supplementary table showing primer sequences are available with the online version of this paper.

nuclease, RNase E (Massé *et al.*, 2003; Morita *et al.*, 2005). Further molecular functions of Hfq include stability control of mRNAs by regulation of polyadenylation (Hajnsdorf & Régnier, 2000; Folichon *et al.*, 2003, 2005; Mohanty *et al.*, 2004) and sequestering RNase E cleavage sites (Folichon *et al.*, 2003; Moll *et al.*, 2003), thereby influencing poly(A)-dependent RNA turnover and endonucleolytic restriction, respectively. Hfq seems to contribute to overall translational performance by accelerating the activity of the tRNA nucleotidyltransferase (Scheibe *et al.*, 2007) and it also binds tRNAs (Lee & Feig, 2008).

Genetic inactivation of *hfq* in diverse eubacteria caused pleiotropic physiological effects (Tsui *et al.*, 1994; Muffler *et al.*, 1996, 1997) and revealed a fundamental role of Hfq in the virulence of pathogenic bacteria (Robertson & Roop, 1999; Sonnleitner *et al.*, 2003; Christiansen *et al.*, 2004; Ding *et al.*, 2004; Sharma & Payne, 2006; Sittka *et al.*, 2007; Wilson *et al.*, 2007; Lucchetti-Miganeh *et al.*, 2008). In this context, there are several reports on Hfq-mediated adaptation to changing environmental conditions, exemplified by *Brucella abortus* (Robertson & Roop, 1999; Gee *et al.*, 2005; Valderas *et al.*, 2005), *Salmonella typhimurium* (Sittka *et al.*, 2007) and *Azorhizobium caulinodans*, as well as the phototrophs *Rhodobacter capsulatus* (Kaminski *et al.*, 1994; Kaminski & Elmerich, 1998; Drepper *et al.*, 2002) and *Rhodobacter sphaeroides* (Glaeser *et al.*, 2007). In contrast, no apparent phenotype emerged from an *hfq* knockout in *Staphylococcus aureus* (Bohn *et al.*, 2007). Although not all eubacterial genomes encode an Hfq protein, an Hfq-like protein has recently been identified in Archaea (Nielsen *et al.*, 2007). Importantly, although this *Methanocaldococcus jannaschii* protein differs from the well-characterized eubacterial Hfq proteins in length and amino acid sequence, it does restore complex phenotypes to an *E. coli hfq* deletion strain (Nielsen *et al.*, 2007).

Initial attempts to explore microbial genomes for Hfq homologues failed with regard to cyanobacteria (Sun *et al.*, 2002). However, broadening the criteria and specifically targeting proteins with a possible Sm motif resulted in the identification of candidate genes in a variety of cyanobacteria (Valentin-Hansen *et al.*, 2004; see Supplementary Fig. S1, available with the online version of this paper). Within the cyanobacterial genus *Prochlorococcus*, *hfq* has been lost in several of the sequenced strains (Axmann *et al.*, 2005). Nevertheless, the naturally *hfq*-deficient *Prochlorococcus* MED4 expresses at least 21 different non-coding RNAs (Axmann *et al.*, 2005; Steglich *et al.*, 2008). Therefore, the functional importance of Hfq in cyanobacteria has remained entirely unclear and has not been experimentally addressed thus far.

Here we show that the *hfq* candidate gene in *Synechocystis* sp. PCC 6803 can be knocked out without any detrimental effects on cell growth. However, the most striking change, which could be completely reversed by reintroduction of the wild-type (WT) gene, is the loss of motility correlated with the absence of type IV 'thick' pili on the cell surface. Microarray analyses suggest a number of genes that depend on Hfq; among them, there are two operons that are controlled by the cAMP receptor protein (CRP) SYCRP1, an established regulator of cell motility, as well as several genes with unknown function and the chaperone genes *groES* and *groEL*.

METHODS

Bacterial strains and growth conditions. The motile strain of *Synechocystis* sp. PCC 6803 used in this study was originally obtained from S. Shestakov (Moscow State University, Russia) and propagated on BG11 agar plates (Rippka *et al.*, 1979; 0.75 %, w/v, agar). Liquid cultures of *Synechocystis* sp. PCC 6803 WT and mutant strains were grown at 28 °C in BG11 medium containing 10 mM TES buffer (pH 8.0) under continuous illumination with white light of 75 μmol photons m^{-2} s^{-1} and a continuous stream of air. For the mutants described below, antibiotics were used at the following concentrations: chloramphenicol (Cm), 7 μg ml^{-1}; kanamycin (Km), 80 μg ml^{-1}.

E. coli strain DH5α was used for all plasmid constructions; strain J53 was used for conjugation with *Synechocystis* sp. PCC 6803. *E. coli* cultures were generally grown in LB medium, supplemented with antibiotics at standard concentrations as appropriate.

Plasmids and mutagenesis. For insertion mutagenesis, the *hfq* ORF [locus *ssr3341* (Kaneko *et al.*, 1996)] was amplified as a 492 bp fragment including 100 bp of the 5′- and 179 bp of the 3′-flanking regions (putative promoter and terminator regions, respectively) using primers P1 and P2 (see Supplementary Table S1, available with the online version of this paper) and ligated into the pGEM-T-Easy vector (Promega). Eventually, the chloramphenicol resistance cassette from pACYC184 (New England Biolabs) was inserted into an *Xma*JI site within *ssr3341* (Fig. 1) which had been converted to blunt ends using the Klenow fragment. This construct was used to transform *Synechocystis* as described by Ermakova *et al.* (1993). Transformants were restreaked six times and analysed by Southern blot hybridization and PCR to detect the level of segregation of WT genome copies.

Complementation of the Δ*hfq* disruption was initially approached by amplification using primers P3 and P4 of an 1111 bp fragment, including *ssr3341*, flanked by 500 bp in the 5′- and 398 bp in the 3′-direction (including its own putative promoter and terminator regions). The amplicon was ligated into the pDrive vector (Qiagen), excised by *Pst*I/*Sal*I digestion and finally inserted into the conjugative, self-replicating vector pVZ321 (Zinchenko *et al.*, 1999), replacing the plasmid's Cm resistance cassette. The resulting plasmid, pVZ-Phfq15, was transferred to Δ*hfq* mutant cells by conjugation (Zinchenko *et al.*, 1999), whereupon exconjugants were selected on BG11 agar containing up to 80 μg Km ml^{-1} and 7 μg Cm ml^{-1}; the latter one due to the insertion in the disruptant background. Mutants engineered with the equivalently constructed plasmid pVZ-hfqS2, which contained a single point mutation at position 121 in the *hfq* coding sequence which leads to an in-frame stop codon and thus to a truncated polypeptide (see Supplementary Fig. S1), served as a negative control for complementation experiments.

Motility assays. Phototactic movement was examined as described by Wilde *et al.* (2002) using BG11 0.5 % (w/v) agar plates supplemented with 10 mM TES (pH 8.0) and 10 mM glucose. The plates were put into opaque boxes with an open slit (3 cm in width) and illuminated with white light of 1–3 μmol photons m^{-2} s^{-1} at 28 °C. Phototactic movement was documented after 2 weeks using a Plustek OpticPro ST48 Scanner.

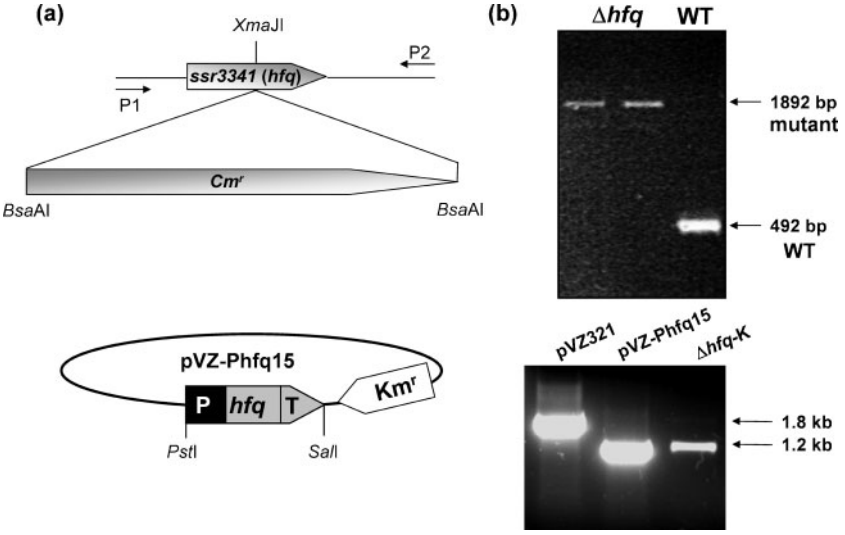

Fig. 1. Inactivation of *hfq* in *Synechocystis* sp. PCC 6803 and complementation. (a) Schematic representation and inactivation strategy of the *Synechocystis* sp. PCC 6803 locus *ssr3341* corresponding to the *hfq* homologue (top); schematic map of the conjugative plasmid pVZ-Phfq15 used for complementation of the Δ*hfq* mutant (bottom). Arrow-shaped boxes, representing the genes/gene cassettes, indicate the direction of transcription. Small arrows show the relative positions of primers used for PCR. Relevant restriction sites are indicated. Cm^r, Cm resistance cartridge; Km^r, Km resistance cartridge; P, putative promoter for *hfq* transcription; P1, Primer hfq-fw; P2, Primer hfq-rev; T, putative terminator of *hfq* transcription. (b) Complete segregation of WT genome copies in two different clones of Δ*hfq* (top) and the presence of the self-replicating complementation plasmid pVZ-Phfq15 in the reverted mutant Δ*hfq-K* (bottom) were verified by colony PCR using the mutagenesis primers and pVZ321-directed primers, respectively. Plasmids pVZ321 and pVZ-Phfq15 were used as control templates. Fragment sizes are indicated. WT, wild-type.

RNA isolation and Northern hybridization. Exponentially growing *Synechocystis* sp. PCC 6803 liquid cultures (OD_{750} 0.6–0.8, Ultrospec III, Pharmacia) were collected by quenching on ice and immediate centrifugation at 0–4 °C. Cell pellets were resuspended in 1 ml TRIzol reagent (Invitrogen) per 20 ml aliquot and total RNA was isolated by following the manufacturer's instructions. The purified RNA was quantified using a NanoDrop ND-1000 spectrophotometer (peqLab Biotechnology) separated by electrophoresis on 1.3 % agarose formaldehyde gels and blotted onto Roti-Nylon plus (Roth) or Hybond-N^+ (GE Healthcare) membranes (Sambrook & Russell, 2001). Hybridization probes were generated either by random prime labelling (Rediprime II labelling kit, GE Healthcare) of PCR products with [α-^{32}P]dCTP (Hartmann Analytic) or by *in vitro* transcription of PCR fragments from the T7 promoter in the presence of [α-^{32}P]UTP (Hartmann Analytic) using the T7 polymerase Maxiscript kit (Ambion). Following prehybridization for at least 30 min in 50 % deionized formamide, 7 % SDS, 250 mM NaCl and 120 mM sodium phosphate pH 7.2 at 55 °C for DNA probes and 68 °C using riboprobes, the labelled probes were added and hybridized for 3–16 h. The membranes were rinsed in 2 × SSC /0.5 % SDS and washed in two subsequent 15 min steps at 68 °C in 2 × SSC/0.5 % SDS and 0.1 × SSC/0.1 % SDS, respectively. Signals were detected and analysed on a Personal Molecular Imager FX system with Quantity One software (Bio-Rad). All DNA oligonucleotides are listed in Supplementary Table S1.

Transmission electron microscopy. Samples of the different *Synechocystis* strains were collected from colonies on the surface of the agar plates by resuspending in BG11 medium. For electron microscopy, the cyanobacteria were negatively stained with 0.5 % aqueous uranyl acetate (Golecki, 1988). Micrographs were taken with a Philips CM10 electron microscope (Fei Company) operating at 80 kV, with integrated BioScan camera model 792 and Digital Micrograph software (Gatan).

Microarray analysis. Microarray experiments were carried out as two-colour hybridizations on custom-designed 8-pack 15K *Synechocystis* sp. microarrays (AMADID 016989) from Agilent Technologies. The RNA integrity and the amount of total RNA were measured with a Bioanalyser 2100 (Agilent Technologies). Per sample, 3 μg total RNA was reverse transcribed with CyScribe post-labelling kit (GE Healthcare). First-strand cDNA probe (300 ng) was labelled by the 'post-labelling' (amino allyl) method with Cy3 and Cy5. Labelling efficiencies and cDNA amounts were determined with a Nanodrop ND-1000 spectrophotometer (Kisker). In order to compensate specific effects of the dyes and to ensure statistically relevant data analysis, a colour-swap dye-reversal was performed (Churchill, 2002). Labelled samples were mixed, hybridized to 15K custom-made microarrays and washed according to the supplier's protocol (Agilent Technologies). Scanning of microarrays was performed with 5 μm resolution using a DNA microarray laser scanner (Agilent Technologies). Features were extracted with an image analysis tool version A.9.5.3 using the GE2-V5_95_Feb07 protocol (Agilent Technologies). Data analysis was carried out on the Rosetta Inpharmics platform Resolver Version 7.0. Ratio profiles were combined in an error-weighted fashion with Resolver to create ratio experiments. A twofold change expression cut-off for ratio experiments was applied together with anti-correlation of ratio profiles, rendering the microarray analysis set highly significant ($P>0.05$), robust and reproducible.

RESULTS

Inactivation of *ssr3341* in *Synechocystis* sp. PCC 6803 and growth characteristics of the deletion strain

To investigate the function of a previously predicted cyanobacterial Hfq orthologue (Valentin-Hansen *et al.*,

2004), we constructed a Δ*ssr3341* mutant in *Synechocystis* sp. PCC 6803 by insertion mutagenesis (from here on designated Δ*hfq*). Complete genomic segregation was verified by Southern blot hybridization (data not shown) and PCR analyses (Fig. 1). In order to assess possibly altered growth characteristics arising from the knockout, WT and mutant strains were grown simultaneously in BG11 liquid medium or on solid agar plates. Neither standard growth conditions nor exposure to different light conditions led to a significantly impaired growth behaviour of the Δ*hfq* mutant (data not shown).

Motility analysis and cell surface images

Phototactic motility of the Δ*hfq* knockout strain was assayed in comparison with the WT under unidirectional white light. WT cells moved towards the light source, whereas Δ*hfq* mutant colonies showed no motility, as evaluated from the smooth edges of the spot (Fig. 2), within which the mutant cells were evenly distributed. Because loss of phototactic motility may arise from the absence of motility-mediating (thick) type IV pili and from hyperpiliation as well (Bhaya *et al.*, 2000), mutant and WT cells were subjected to electron microscope examinations. Whereas WT cells possess mainly two types of pili on their surface, thin pili and thick pili (Bhaya *et al.*, 2000; Yoshihara *et al.*, 2001), piliation appeared to be completely absent from Δ*hfq* mutant cells (Fig. 3b). In order to exclude possible secondary effects caused by the inserted resistance cassette or by unrelated mutations, we restored the WT phenotype (i.e. motility) by introducing a complementing *hfq* copy into the mutant cells. Since the absence of pili was also accompanied by a loss of natural competence for transformation (data not shown) we used the conjugative shuttle vector pVZ-Phfq15, harbouring the *hfq* gene flanked by its own promoter and terminator regions for complementation. Additionally, conjugation was performed with the negative control vector pVZ-hfqS2, containing the same DNA fragment with a single point mutation in the *hfq* gene leading to a truncated ORF (Supplementary Fig. S1). The motility analysis (Fig. 2) including the resulting strains *hfq-K* (complementation) and *hfq-S* (negative control) demonstrated that the introduction of an independent, extrachromosomal WT copy of *hfq* reverted the mutant back to the WT phenotype. Cells containing the original pVZ321 vector (Zinchenko *et al.*, 1999) apparently slowed down the movement of the cells towards the light source. This effect is assumed to be a consequence of a shifted cellular energy balance caused by the additional replicon and the accompanied expression of foreign protein (Bentley *et al.*, 1990). The *hfq-S* strain harbouring the truncated version of *hfq* did not move towards the light, indicating that the non-motile phenotype was exclusively due to the loss of Hfq. In accordance, the genetic complementation restored the piliation on the mutants' cell surface, as is shown in Fig. 3(c).

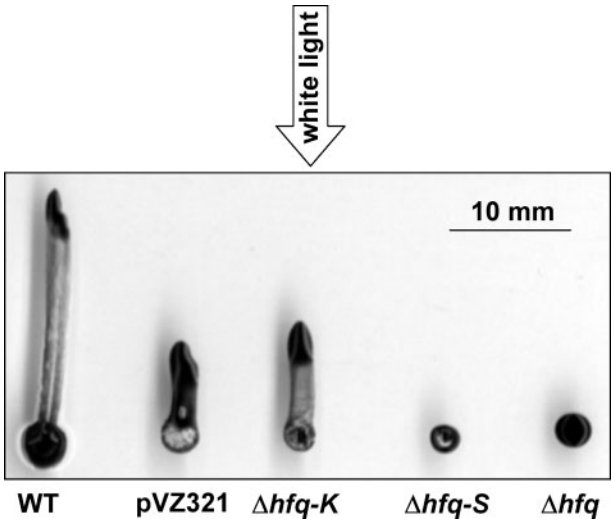

Fig. 2. Directional motility assay. Exponentially growing cells (OD_{750} ~0.4) were spotted onto a 0.5% agar BG11 plate without antibiotic supplements and exposed to an unidirectional light source (arrow) for 2 weeks. WT, wild-type; pVZ321, WT strain harbouring the unmodified pVZ321 plasmid; Δ*hfq*, disruptant; Δ*hfq-K*, complemented strain harbouring pVZ-Phfq15; Δ*hfq-S*, negative control strain harbouring pVZ-hfqS2, containing an internal stop codon within the *hfq* ORF.

Screening target genes for Hfq using DNA microarray analysis

To analyse the effects of the *hfq* disruption on gene expression, microarrays that cover 3264 protein-coding genes of the *Synechocystis* sp. PCC 6803 genome (Eisenhut *et al.*, 2007) were hybridized with total RNA from two independent preparations of WT and mutant cells. Thirty-one genes displayed statistically significant changes in transcript levels in the Δ*hfq* knockout background (Table 1). Intriguingly, these genes emerged as being almost exclusively repressed in the disruptant, while only two genes (*ssl3445* and *sll1942*) showed an inverse behaviour. Among the most strongly responding genes were *slr1667*, *slr1668*, *slr2015*, *slr2016* and *slr2018*, which had previously been described as five of six target genes for SYCRP1, a cyanobacterial CRP, encoded by *sll1371* (Yoshimura *et al.*, 2002a, b). The microarray analysis revealed several additional genes whose expression was significantly repressed in the Δ*hfq* mutant that are also located in tandem on the chromosome. Besides *slr2075* and *slr2076*, encoding the chaperonins GroEL and GroES, respectively, expression of *slr0150* and *slr0151*, the former encoding the ferredoxin PetF, appears to be co-regulated in an Hfq-dependent manner. In addition, transcript accumulation of the gene pairs *sll1429–sll1430* and *sll1239–sll1240* as well as of several single genes (Table 1) was reduced in the Δ*hfq* mutant. However, apart from the two SYCRP1-regulated operons, no functions have yet been assigned to most of these putative Hfq targets.

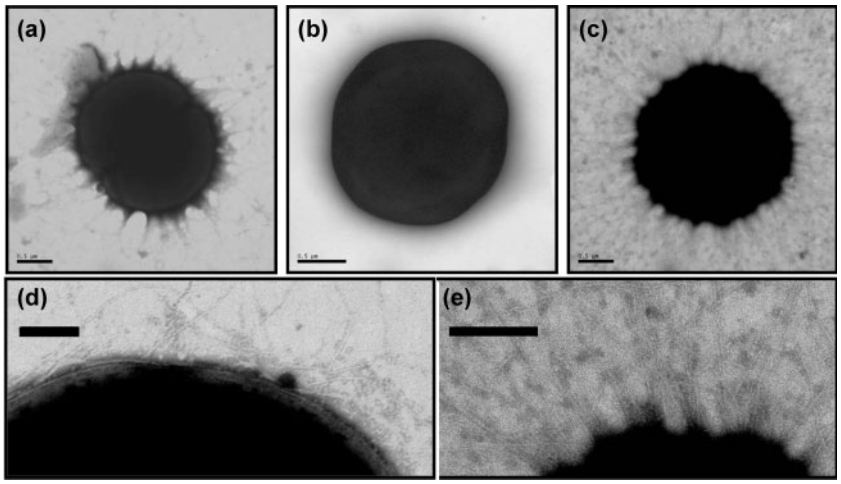

Fig. 3. Electron micrographs of negatively stained *Synechocystis* sp. PCC 6803 WT and mutant cells. (a) Typical WT cell with type IV pili. (b) Δ*hfq* mutant showing loss of piliation. (c) Revertant Δ*hfq-K* showing recovered piliation. (d, e), WT and Δ*hfq-K* cells, respectively, at higher magnification (46 000×). Bar, 0.5 μm for (a), (b), (c), (e); 0.2 μm for (d).

Verification of the microarray data by Northern blot analyses

The results of the microarray experiments were verified by Northern blot analyses using hybridization probes for the initial and terminal genes in operons *slr1667–slr1668* and *slr2015–slr2016–slr2017–slr2018*. As *ssr2848* (encoding a hypothetical protein) appeared to be the second most affected gene in the microarray analysis, its differential expression was also chosen to be verified, along with *slr1764*, which has been annotated as a cAMP-binding protein, TerE. Fig. 4 shows confirmation that transcript accumulation of representatives of both the above-mentioned operons is drastically reduced in Δ*hfq*. In particular, *slr1667* and *slr1668* mRNAs were not detectable in the mutant by Northern blot hybridization, suggesting a complete repression of these genes owing to the absence of Hfq (Fig. 4a). The same conclusion can be drawn from the probing of *ssr2848* and *slr1764* (Fig. 4b), again supporting the validity of the dataset. Furthermore, the expression levels of *slr2015* and *slr2018*, as detected by Northern blot analysis, corresponded with the microarray data: each gene showed a rather moderate repression (Fig. 4a).

The photosynthesis-related transcript *psbA2* was hybridized as a control, demonstrating that the observed effects were not based on a global, rather non-specific shut-down of gene expression in the Δ*hfq* disruptant (Fig. 4b).

Three further genes were probed that are known to be essentially involved in cellular motility (Kaimei *et al.*, 2001; Yoshihara *et al.*, 2001; Yoshihara & Ikeuchi, 2004; Panichkin *et al.*, 2006). Unlike the microarray dataset, the Northern blot data revealed that *pilA1*, encoding the main structural component of type IV pili, showed a slight decrease in transcript abundance in Δ*hfq*. In contrast, transcript accumulation of *pilB1* and *spkA*, encoding a nucleoside triphosphatase (NTPase) and a serine/threonine kinase, respectively, was increased in the mutant (Fig. 4b, c). Further, Northern blot hybridizations of total RNA isolated from the complementation strain Δ*hfq-K* and the negative control strain Δ*hfq-S*, applying the *slr1667* probe, demonstrated the restoration of the WT phenotype at the molecular level (Fig. 5). Thus, these data clearly show Hfq-dependent regulation of motility-related genes and operons as well as of several further genes, whose function remains to be elucidated.

DISCUSSION

The *Synechocystis* sp. PCC 6803 Hfq candidate protein is only 70 aa in length and its sequence is rather diverse compared with functionally characterized RNA chaperones. Yet the severe phenotypic effects and changes in mRNA abundances observed here resemble Δ*hfq* mutants in other model organisms and therefore provide circumstantial evidence that *ssr3341* does encode a cyanobacterial Hfq protein. *Synechocystis* sp. PCC 6803 is able to move towards light sources in a process called twitching motility (Bhaya, 2004) that might enhance the fitness of cyanobacterial cells in biofilms, especially with regard to diurnal changes in illumination (for reviews on motility with regard to biofilm formation and light adaptation see Bhaya, 2004; Nudleman & Kaiser, 2004). The central morphological prerequisite for this phototactic movement is the presence of type IV pili (McBride, 2001), which are also known to be essential for their natural competence (Yoshihara *et al.*, 2001; Nakasugi *et al.*, 2006). Both of these pili-dependent mechanisms were lost as a consequence of *hfq* inactivation in *Synechocystis* sp. PCC 6803, and electron microscopy provided evidence for the loss of type IV pili on the mutants cell surface. Targeting molecular details for the non-piliated Δ*hfq* phenotype, microarray analysis revealed the two operons *slr1667–slr1668* and *slr2015–2018* to be most strongly repressed in the Δ*hfq* mutant compared to WT cells. The decrease in transcript levels of *slr1667–slr1668* in Δ*hfq* is comparable to that previously observed in a disruption mutant of *sycrp1* (Yoshimura *et al.*, 2002a; Panichkin *et al.*, 2006). This gene

Table 1. Differentially expressed genes in *Synechocystis* sp. PCC 6803 WT and Δ*hfq* mutant as determined by microarray analysis

Genes subjected to Northern blot verification are in bold type.

Genomic locus	Description	Fold change*
slr1667	**Target of SYCRP1**	**−48.84**
slr1668	**Target of SYCRP1**	**−29.10**
ssr2848	**Unknown**	**−12.57**
sll1543	Hypothetical protein	−6.08
ssr2787	Unknown	−5.97
slr2016	PilA10	−5.53
ssl2996	Unknown	−5.36
slr2018	**Unknown**	**−4.49**
sll1514	HspA	−4.11
slr2015	**PilA9**	**−4.03**
slr1764	**cAMP-binding protein**	**−3.42**
slr0442	Unknown	−3.30
slr0120	tRNA/rRNA methyltransferase	−3.07
slr2076	GroEL	−3.06
slr2075	GroES	−2.95
sll1239	Unknown	−2.96
sll0910	TPR unknown	−2.85
slr0397	Hypothetical protein	−2.82
sll0711	Isopentenyl monophosphate kinase	−2.78
sll0377	Transcription repair coupling factor	−2.77
sll0428	Unknown	−2.63
sll1366	Putative SNF2 helicase	−2.57
sll1429	Unknown	−2.49
slr0151	Unknown	−2.43
sll1240	Unknown	−2.40
slr1917	Hypothetical protein	−2.39
slr0106	Unknown	−2.27
sll1430	Adenine phosphoribosyltransferase	−2.31
slr0150	Ferredoxin, PetF-like protein	−2.14
ssl3445	50S ribosomal protein L31 (rpl31)	2.29
sll1942	Unknown	2.44

*The fold change values represent the average of two independent experiments, which were each technically duplicated and additionally subjected to a dye swap. Changes in gene expression less than +/− twofold were not considered to be significant.

encodes the CRP SYCRP1 which binds to the promoter region of *slr1667* and directly controls its expression as a transcriptional factor (Yoshimura *et al.*, 2002a). These facts suggest an interesting relationship between cAMP signalling, motility and possible RNA-based regulation, since disruption of *sycrp1* resulted in a non-motile phenotype as well (Yoshimura *et al.*, 2002b). There are different reports suggesting an important role for the second messenger cAMP in motility of *Synechocystis* sp. PCC 6803 cells (Terauchi & Ohmori, 1999, 2004). Mutant colonies lacking the adenylate cyclase Cya1 were impaired in forming typical finger-like projections during phototactic movement, whereas motility was not affected at the individual cell level (Bhaya *et al.*, 2006). The former could be reversed by the addition of cAMP to the medium, though not leading to the restoration of motility in *sycrp1* mutant cells. Similarly, in this work, non-motile Δ*hfq* mutant cells were also not rescued by cAMP addition (data not shown). However, there seems to be an important difference between Δ*sycrp1* and Δ*hfq* mutant cells, that is to say motility of *sycrp1* disruptants is affected only in the 'strong' phototaxis characterized by projections on the agar plate, while individual cells still appear motile (Bhaya *et al.*, 2006).

Here, it is interesting to draw a comparison with enterobacteria, since the cAMP–CRP complex in *E. coli* is required for flagellum synthesis, besides its involvement in the regulation of several catabolic functions (Botsford & Harman, 1992). Moreover, transcription of the Hfq-dependent sRNAs Spot42 and CyaR in *E. coli* and *Salmonella*, respectively, is controlled by the cAMP–CRP complex (Polayes *et al.*, 1988; Papenfort *et al.*, 2008); there is evidence that *cyaR* transcription is under direct control of cAMP and CRP (Papenfort *et al.*, 2008). However, given that cAMP supply could not restore motility in *Synechocystis* sp. PCC 6803 Δ*hfq*, we suppose that Hfq acts epistatically to cAMP-SYCRP1 and probably interferes with a very late step of a signalling chain that regulates cellular movement.

Another interesting aspect arising from the Northern blot data is the apparent involvement of Hfq in accumulation of *spkA* and *pilB1* mRNAs, encoding a serine/threonine protein kinase and a putative ATPase, respectively, and also in control of the abundance of *pilA1*, encoding the major structural component of pili; all these genes are essential for motility (Kamei *et al.*, 2001; Yoshihara *et al.*, 2001; Panichkin *et al.*, 2006). In line with the chosen expression cut-off and statistical stringency, these genes were not identified in the course of the microarray analysis. Interestingly, we found that both *spkA* and *pilB1* became moderately activated in the absence of Hfq, whereas a decrease in *pilA1* expression levels in the Δ*hfq* mutant was seen. Intriguingly, the inverse change in expression levels of *pilA1* and *spkA* in Δ*hfq* complies with the observation that *pilA1* expression is enhanced in a Δ*spkA* mutant (Panichkin *et al.*, 2006). In contrast, the expression level of the *slr2015–2018* operon was lowered in both Δ*hfq* and Δ*spkA* mutants (Panichkin *et al.*, 2006), together suggesting that the Hfq-dependent signalling pathway is different from SpkA-dependent motility control.

Hfq also appears to be involved in the assembly and functionality of extracellular appendages in other groups of bacteria. We note that there is – irrespective of the completely different lifestyles of pathogens and cyanobacteria – an analogy between pathogenicity and (phototactic) motility in terms of the dependence of both phenomena on such cellular appendages and adherence (reviewed by Bhaya, 2004; Nudleman & Kaiser 2004; Pizarro-Cerdá & Cossart, 2006). Although cell surfaces were not further examined, twitching and swarming mediated by type IV pili were found to be impaired in a Δ*hfq* mutant of

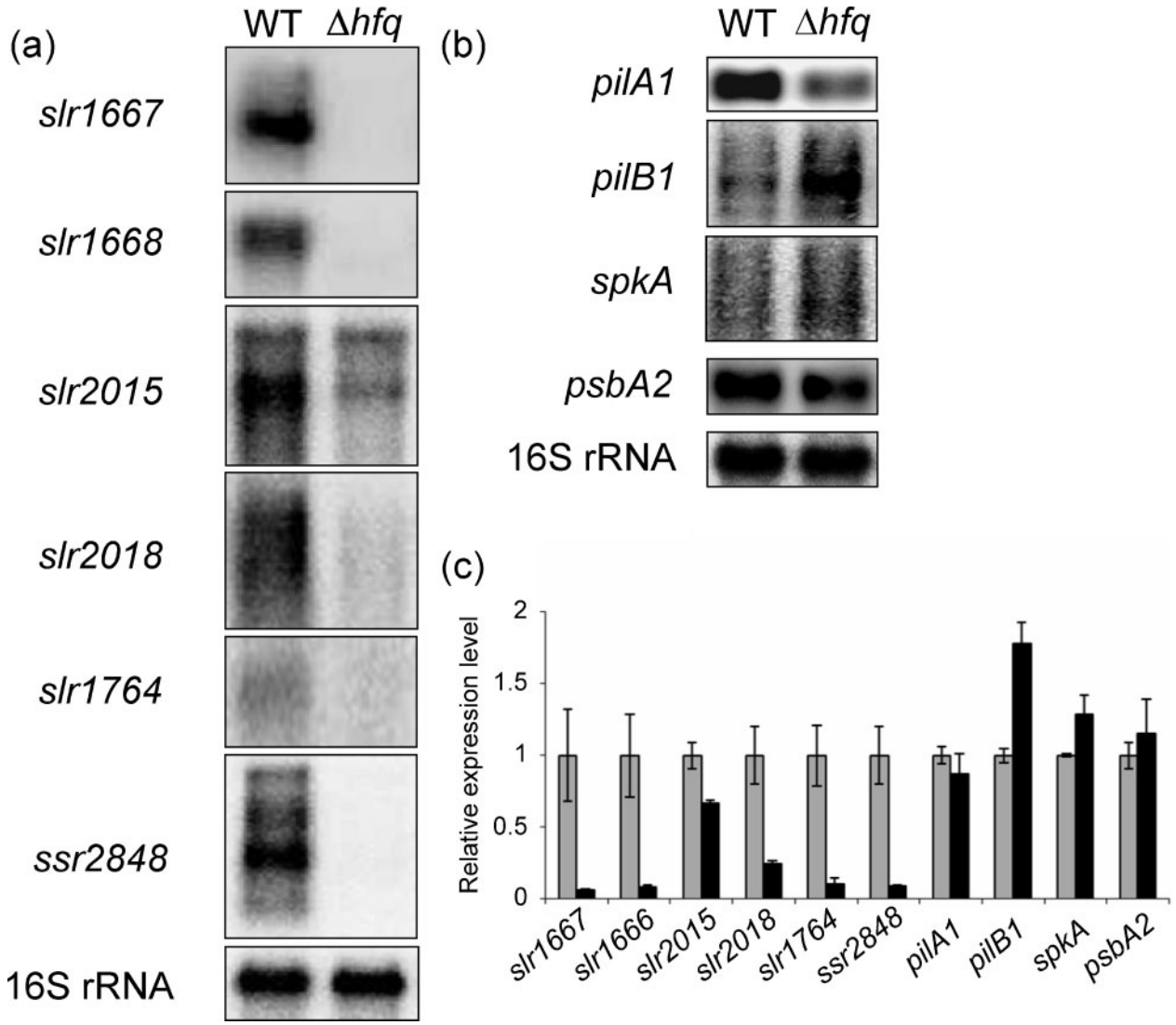

Fig. 4. Confirmation of microarray analysis by representative Northern blot experiments and semiquantitative analysis. Total RNA was isolated from WT and mutant cells (Δ*hfq*) and samples (5 μg) were separated as described in Methods. (a) Exemplary verification of altered expression levels of the two motility-related operons *slr1667-slr1668* and *slr2015–slr2016–slr2017–slr2018* (represented by *slr2015* and *slr2018*) due to the *hfq* inactivation, as well as altered mRNA concentrations of the two additional genes, *slr1764* and *ssr2848*. (b) Hybridization of the piliation and motility-related genes *pilA1*, *pilB1* and *spkA*, and of *psbA2* as negative control to exclude an overall repression of gene expression in the Δ*hfq* mutant. (c) Histogram representing semiquantitative densitometric analyses of each selected mRNA signal normalized to the corresponding 16S rRNA signal. The expression levels in Δ*hfq* (black bars) are each relative to the standardized WT levels (grey bars). Data shown are means ± SD from three experiments.

Pseudomonas aeruginosa (Sonnleitner *et al.*, 2003). In a Δ*hfq* mutant of *Salmonella typhimurium*, impaired cell motility correlated with a reduction of flagellin (FliC) expression and drastically reduced virulence (Sittka *et al.*, 2007). However, the structural and mechanistic features of this type of dynamic cell surface appendages are completely different from those of cyanobacterial type IV pili (Bardy *et al.*, 2003). Hence, the functions of the cyanobacterial Hfq orthologue cannot readily be inferred from data for other bacteria.

Certainly, sRNAs are frequently involved in Hfq-dependent regulatory processes in bacteria (reviewed by Storz *et al.*, 2004; Gottesman, 2005; Majdalani *et al.*, 2005), and here we provide the first support for the idea that riboregulatory processes play a role in the control of motility in cyanobacteria. Nevertheless, at present we do not have a clear indication of an RNA chaperone activity of this cyanobacterial Hfq homologue, or of the involvement of a particular sRNA in the regulation of motility. The changes in mRNA levels observed here upon inactivation of *hfq*

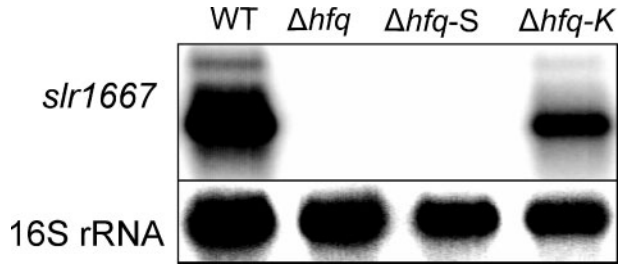

Fig. 5. Northern blot-based verification of the genetic complementation of the *hfq* disruptant. Total RNA was isolated from exponentially growing cultures of the WT and three different mutant strains: Δ*hfq*, disruptant; Δ*hfq-K*, complemented strain harbouring pVZ-Phfq15; Δ*hfq-S*, negative control strain harbouring pVZ-hfqS2, containing an internal stop codon within the *hfq* ORF. As the *hfq* knockout had the most striking effect on *slr1667* expression, this one was chosen as the representative of *hfq*-regulated genes.

may not only result from direct binding of Hfq or from disturbed interaction with unknown sRNAs. A comparison of the transcriptome in a *Salmonella hfq* mutant with the set of Hfq-associated mRNAs has recently shown that the altered transcriptome profile of the mutant can, to some extent, be attributed to secondary effects of the deregulation of transcription factors whose synthesis is directly regulated by Hfq (Sittka *et al.*, 2008).

Preliminary results on *isiA* and its regulatory antisense-RNA IsrR (Dühring *et al.*, 2006) argue against a role of the cyanobacterial Hfq in riboregulation of photosynthesis; neither of these two transcripts was altered in the Δ*hfq* mutant (data not shown). Arguably, RNA binding remains to be shown for the cyanobacterial Hfq homologue, and associated RNAs with functions in motility control remain to be identified. One strategy that could be used to address these two questions in parallel could be Hfq co-immunoprecipitation, followed by the identification of associated sRNAs (and putative target mRNAs) by microarray analysis (Zhang *et al.*, 2003), direct sequencing (Christiansen *et al.*, 2006) or high-throughput cDNA sequencing (Sittka *et al.*, 2008).

Notwithstanding, the signalling pathway that controls motility in *Synechocystis* sp. PCC 6803 is clearly more complex than previously thought, integrating Hfq as a new factor. Thus, the functional mechanism(s) of Hfq action in *Synechocystis* sp. PCC 6803, with special regard to cAMP signalling and motility, as well as potential interaction partners, remains to be investigated and will provide an exciting research area for the future.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft Focus program 'Sensory and regulatory RNAs in Prokaryotes' SPP1258 (projects HE 2544/4-1, WI 2014/2-1 and WI 2014/3-1). We thank Gisa Baumert, Ina Wagner, Nils Schürgers, Ingeborg Scholz, Jens Georg and Thomas Wallner for excellent technical assistance or for providing cultures for electron microscopy and Hans Mattijs for access to the microarray design.

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Edited by: D. J. Scanlan