Bacillus subtilis Hfq: A role in chemotaxis and motility

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Hfq is a global post-transcriptional regulator that modulates the translation and stability of target mRNAs and thereby regulates pleiotropic functions, such as growth, stress, virulence and motility, in many Gram-negative bacteria. However, comparatively little is known about the regulation and function(s) of Hfq in Gram-positive bacteria. Recently, in *Bacillus subtilis*, a role for Hfq in stationary phase survival has been suggested, although the possibility of Hfq having an additional role(s) cannot be ruled out. In this study we show that an ortholog of Hfq in *B. subtilis* is regulated by the stress sigma factor, σ^B, in addition to the stationary phase sigma factor, σ^H. We further demonstrate that Hfq positively regulates the expression of flagellum and chemotaxis genes (*fla/che*) that control chemotaxis and motility, thus assigning a new function for Hfq in *B. subtilis*.

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1. Introduction

Hfq in *Escherichia coli* and other Gram-negative bacteria acts as a global post-transcriptional regulator controlling translation and stability of target mRNAs (Møller *et al*. 2002; Storz *et al*. 2004; Valentin-Hansen *et al*. 2004). In many Gram-negative bacteria, Hfq has been shown to be involved in the regulation of functions such as stress resistance (Tsui *et al*. 1994; Christiansen *et al*. 2004; Yang *et al*. 2010; Chambers and Bender 2011), virulence (Christiansen *et al*. 2004; Sittka *et al*. 2007; Geng *et al*. 2010; Schiano *et al*. 2010) and motility (Sonnleitner *et al*. 2003; Ding *et al*. 2004; Sittka *et al*. 2007). However, Hfq in Gram-positive bacteria, except in *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium difficile*, is still poorly characterized. In *S. aureus*, an *hfq* mutant showed increased carotenoid pigmentation and decreased virulence. In *L. monocytogenes*, Hfq appears to play a role in pathogenecity in mice, tolerance to osmotic and ethanol stress, and is involved in the long-term survival of the bacteria under amino-acid-limiting conditions (Christiansen *et al*. 2004; Liu *et al*. 2010). In *C. difficile*, Hfq depletion led to pleiotropic effects such as morphological changes, increased sensitivity to stresses, greater ability to sporulate as well as formation of biofilms (Boudry *et al*. 2014).

In *E. coli, hfq* is organized as a seven-gene operon that is regulated by three σ^{32}-dependent promoters and four σ^{70}-dependent promoters. σ^{32} is a heat shock sigma factor that regulates the expression of genes under stress such as growth at high temperature. The presence of multiple σ^{32}-dependent promoters ensures optimum level of Hfq during stress (Tsui and Winkler 1994). Likewise, in *L. monocytogens* as well, *hfq* is regulated by a σ^B-dependent promoter that is induced during various stress conditions, including osmotic and ethanol stress and at the entry into the stationary growth phase (Christiansen *et al*. 2004).

B. subtilis is the best-studied bacterium after *E. coli*, and yet there is very little information available about the regulation of *hfq* in this organism. In *B. subtilis*, *hfq* appears to be organized in an operon with two genes, *miaA*, encoding for a tRNA modification enzyme and *ymaF*, a gene of unknown function, located upstream (figure 1A). A transcription terminator has been predicted at the end of the *hfq* gene (*http://genolist.pasteur.fr/SubtiList/*). The short intergenic distances

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of 101 bp and 42 bp between *ymaF* and *miaA,* and *miaA* and *hfq,* respectively, raises the possibility of these being transcribed as a single transcription unit. However, RNA sequencing of *B. subtilis* transcriptome did not show any poly-cistronic transcripts originating from either *ymaF or miaA* genes (Irnov *et al*. 2010; Dambach *et al*. 2013). In contrast, expression profiling by microarray does suggest the co-transcription of *hfq* with the upstream genes (Nicolas *et al*. 2012). These contrasting results could be resolved by a more suitable technique such as reverse transcription PCR (RT-PCR).

The role of Hfq as an RNA chaperon has been questioned in *B. subtilis* and other Gram-positive bacteria (Jousselin *et al*. 2009). However, the presence of *hfq* in many *Bacillus* sp. (Sun *et al*. 2002) suggests the possibility that Hfq does have a role in this group of bacteria. Indeed, deep sequencing of *B. subtilis* Hfq (Hfq_{BS})-associated RNAs revealed its binding with small RNAs, antisense RNAs and leader sequences within mRNAs (Dambach *et al*. 2013), suggesting its probable role in post-transcriptional regulation. Recently, a role for Hfq_{BS} in stationary phase survival has been suggested (Hammerle *et al.* 2014; Rochat *et al.* 2015). Comparative transcriptome studies carried out on wild type and an *hfq* mutant grown in rich and minimal media revealed that hfq_{BS} affects different sets of genes during stationary phase growth in the two conditions (Hammerle *et al.* 2014; Rochat *et al.* 2015). This may well indicate that Hfq in *B. subtilis* may regulate a variety of genes in its natural soil habitat as it has to adapt to changing nutrient compositions. The above observations, thus, suggest additional roles for Hfq in *B. subtilis*.

In *B. subtilis*, many alternative sigma factors are involved in the transcriptional regulation of target genes that contribute to processes such as motility, stress, sporulation (Chen and Helmann 1994; Haldenwang 1995; Höper *et al.* 2005), etc. For instance, sigma factors σ^D and σ^B are primarily responsible for transcription of genes involved in motility/chemotaxis and stress, respectively (Chen and Helmann 1994; Höper *et al.* 2005; Gupta and Rao 2009). Likewise, four alternate sigma factors, σ^E, σ^F, σ^G and σ^K, are required for transcription of genes whose products are involved in sporulation (Haldenwang 1995). This study was aimed at investigating the transcriptional regulation and function of *hfq* in *B. subtilis*. Identification of transcriptional regulators of a gene may provide insight into its possible role in the processes controlled by these regulators. In the present study, we demonstrate that *hfq* in *B. subtilis* is transcribed by a σ^B-dependent promoter as a single transcription unit that includes the two upstream genes, *ymaF* and *miaA* (figure 1A), as well as by a σ^H-dependent promoter that lies within the intergenic region between *miaA* and *hfq*. Our results further show the involvement of Hfq in chemotaxis and motility.

2. Materials and Methods

2.1 *Strains, plasmids and culture media*

Bacterial strains and plasmids used in this study are listed in table 1. *E. coli* DH5α was used as an intermediate host for plasmid constructions. Both *E. coli* DH5α and *B. subtilis* cells were grown at 37°C in LB medium unless specified otherwise. *B. subtilis* cells were transformed with plasmid DNA by competence method (Spizizen 1958). For chemotaxis assay, *B. subtilis* PY79 and PY79Δ*hfq* strains were grown in PRBG broth (Phenol Red Basal broth with glucose; 10 g of peptone, 5 g of sodium chloride, 1 g of beef extract, 20 g of glucose and 0.001 g of phenol red per liter of distilled water, pH 7.2). Chemotaxis assay was carried out in Taxis medium (14 g of K2HPO4, 6 g of KH2PO4, 2 g of (NH4)2SO4, 0.25 g of MgSO4.7H2O, 17 mg of MnSO4.7H2O, 5 g of Glycerol, 1 g of Tween 80 per liter of distilled water, pH 7.0). For swimming motility assay, 0.3% Tryptone agar (1 g of Tryptone, 0.5 g of NaCl, 0.3 g of Agar per 100 mL distilled water) with 2% glucose was used. Wherever necessary, antibiotics were added to the following final concentrations: for *E. coli*, ampicillin was added to a concentration of 100 µg per mL, whereas for *B. subtilis*, kanamycin, chloramphenicol and erythromycin were added to a final concentration of 10 µg, 5 µg and 1 µg per mL, respectively.

RNeasy mini kit (Qiagen GmbH, Hilden, Germany) was used for RNA isolation. RNase-free DNaseI (Ambion, USA) was used for removal of DNA in RNA preparations. Transcriptor cDNA synthesis kit and SYBR Green I Master kit manufactured by Roche Diagnostic GmbH, Germany, were used for reverse transcription and qRT-PCR, respectively. FirstChoice RLM-RACE kit (Ambion, USA) was used for determination of Transcription start site of *hfq* transcripts. Real-time PCR was carried out in LightCycler 480 II system (Roche Diagnostic GmbH, Germany).

2.2 *Construction of Plasmids pYF, pYH and pICY*

For the construction of pYF, a 648 bp DNA fragment carrying a putative σ^B promoter of *ymaF* along with the first 74 codons of *ymaF* ORF was PCR-amplified with primers HFQF4 and HFQR4 (table 2; figure 2A) that contain restriction enzyme sites for *Hin*dIII and *Bam*HI, respectively. The amplified PCR product was digested with *Hin*dIII and *Bam*HI and cloned as a translational fusion with a promoterless *lacZ* in plasmid pRB381 (Bruckner 1992), an *E. coli–B. subtilis* shuttle vector, to yield the plasmid, pYF (table 1). Presence of the cloned fragment was verified by PCR with primers HFQF4 and KKR35 (corresponds to sequence

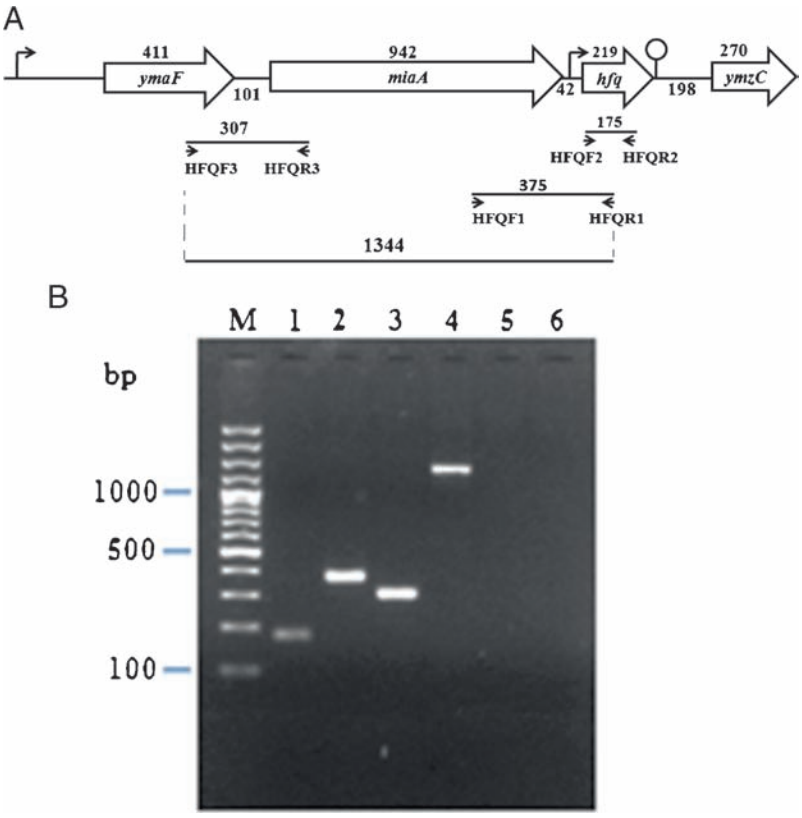

Figure 1. (**A**) Structural organization of *ymaF-miaA-hfq* genes. Numbers above each ORF (block arrows) denotes the length in nucleotides while numbers between ORFs denote the length of the intergenic region. Promoters are shown by bent arrows while terminator is shown with a lollipop structure. Short arrows denote the positions of primers used for RT-PCR analysis and lines linking them indicate the amplification of PCR products of the designated size in base pairs. (**B**) Reverse-Transcription PCR (RT-PCR) analysis of *hfq* expression. RT-PCR was carried out with primers HFQF2/HFQR2 (lane 1), HFQF1/HFQR1 (lane 2), HFQF3/HFQR3 (lane 3) and HFQF3 and HFQR1 (lane 4). Lane 5 shows result for a control in which RNA was treated with RNase A prior to RT-PCR while lane 6 is a no template control. HFQF2/HFQR2 primer pair whose sequences are complementary with *hfq* gene sequence was used in both the control reactions.

within *lacZ* gene) followed by sequencing of the PCR product. pYF was transformed into *B. subtilis* 168 and 1A675, a *sigB* mutant, to obtain the *lacZ* reporter strains 168/YF and 1A675/YF. In parallel, pRB381 was also transformed into 168 and 1A675 to obtain 168/RB381 and 1A675/RB381 that served as control strains.

For the construction of pYH, a 103 bp DNA fragment containing the putative σ^H promoter of *hfq* was amplified by PCR with primers HFQF5 and HFQR5 (table 2; figure 3A). The PCR product was digested with *Bam*HI and *Hin*dIII and cloned adjacent to the *E.coli lacZ* gene in pDG1661, an integrative plasmid (Guerout-Fleury *et al*. 1996), to obtain pYH (table 1). Presence of the 103 bp DNA fragment in the construct was verified by PCR with primers HFQF5 and KKR35 and by sequencing the resultant product. pYH was linearized and transformed in *B. subtilis* 168 and 1S20, a *sigH* mutant, to obtain 168::YH and 1S20::YH, respectively. The plasmid pDG1661 was also transformed in strains 168 and 1S20 to yield 168::DG and 1S20::DG, respectively.

For the construction of pICY, the *hfq* gene with its putative σ^H promoter was amplified with primers HFQF9 and HFQR9 (table 2; figure 3A) and the PCR product was cloned at the *Sal*I restriction site in pIC56 (table 1), a *B. subtilis–E. coli* shuttle vector. The presence of *hfq* gene in pICY was verified by DNA sequencing. pICY was transformed into PY79Δ*hfq* to generate PY79Δ*hfq*/pICY.

2.3 *Assay for β-galactosidase in B. subtilis*

B. subtilis strains carrying *lacZ* constructs were grown at 37°C in Luria Bertani broth containing appropriate antibiotics and incubated in an orbital shaker at 200 r.p.m. Samples were harvested at desired time points and β-galactosidase activities were determined (Nicholson and Setlow 1990). Enzyme

Table 1. Plasmids and strains used in this study

Plasmid or strain	Description or genotype	Source or reference
Plasmids		
pRB381	*E. coli-Bacillus* shuttle vector for translational fusion with β-galactosidase gene; '*lacZ* Km^r Ap^r	BGSC*(Bruckner 1992)
pDG1661	*B. subtilis* integration vector at *amy* locus; '*lacZ* Ap^r Cm^r	BGSC
pIC56	*E. coli-Bacillus* shuttle vector, Ap^r, Em^r	BGSC
pYH	pDG1661 bearing 103 bp insert upstream to *hfq* ORF containing putative σ^H promoter in transcriptional fusion with *lacZ* gene	This study
pYF	pRB381 bearing 648 bp insert containing *ymaF* promoter, RBS and first 74 codons in translation fusion with *lacZ* gene	This study
pICY	pIC56 bearing 559 bp insert containing *hfq* promoter, RBS, ORF and terminator	This study
Strains		
168	*trpC2*	BGSC
1A675	*furB1 sigB::cat trpC2*	BGSC
1S20	*sigH4 str trpC2*	BGSC
PY79	wild type	Youngman *et al*. 1983
PY79Δ*hfq*	*B. subtilis* PY79 *hfq*::*spc*	Silvaggi *et al*. 2005
168/YF	*B. subtilis* 168 bearing pYF	This study
1A675/YF	1A675 bearing pYF	This study
168/RB381	*B. subtilis* 168 bearing pRB381	This study
1A675/RB381	1A675 bearing pRB381	This study
168::YH	168 transformed with pYH (*amy::hfqP-lacZ*, Cm^R)	This study
1S20::YH	1S20 transformed with pYH (*amy::hfqP-lacZ*, Cm^R)	This study
168::DG	168 transformed with pDG1661	This study
1S20::DG	1S20 transformed with pDG1661	This study
PY79Δ*hfq*/pICY	PY79Δ*hfq* mutant transformed with pICY	This study

*BGSC: Bacillus Genetic Stock Centre, Ohio State University, Ohio

activity was expressed in terms of Miller units (MU) (Miller 1992). The activities presented are the mean ± standard deviations of triplicate values. At least three independent experiments were performed and a representative result is shown.

2.4 *Swimming motility assay*

1 μL of *B. subtilis* PY79 and PY79Δ*hfq* cultures grown in PRBG medium were spotted at the centre of 0.3% Tryptone agar plates supplemented with 2% glucose. The plates were incubated at 37°C and observed for surface motility.

2.5 *Chemotaxis assay*

B. subtilis PY79 and PY79Δ*hfq* strains were grown in PRBG broth until stationary phase. Cells were harvested and washed twice with the taxis medium before chemotaxis assay (Drift and Jong 1974). Glucose (10 mM) in Taxis medium was used as an attractant while control capillaries contained only Taxis medium. Bacteria migrating towards attractant due to chemotactic activity were expressed as Chemotactic Number (Kirby *et al.* 2001). The chemotactic number presented is the mean ± standard deviations of triplicate values. At least three independent experiments were performed and a representative result is shown.

2.6 *Reverse-transcription PCR (RT-PCR)*

B. subtilis cells were grown in Luria Bertani (LB) broth till stationary phase (A_{600} ~4.5). Total RNA was isolated with RNeasy mini kit and treated with DNaseI to remove any contaminating DNA. Reverse transcription was carried out at 45°C for 30 min with the Transcriptor cDNA synthesis kit followed by PCR with gene-specific primers as shown in figure 1. RT-PCR products were analysed on a 2% agarose gel and viewed under an UV-Transilluminator.

Table 2. Primers used in this study

Primers	Sequence (5′————3′)†	Restriction site
HFQF1	TTTGTGACACTTTCCGATGC	None
HFQR1	CTGCTTACCTTCCGATTCCA	None
HFQF2	TCAAGGAGGACGAAACATGA	None
HFQR2	AAGCTGCTGCTTACCTTCC	None
HFQF3	GTA CGG AGG GGT TGT GTA CG	None
HFQR3	TTT CCG CGT TTA AGG ATT TG	None
HFQF4	CGTAAAGCTTGAGTCAAGAGATTCATTTTCACA	*Hin*dIII
HFQR4	ATCGGGATCCCCGGTCCTGAGATTCCGTA	*Bam*HI
HFQF5	GTACAAGCTTGATATGACACCGCCTGTTGA	*Hin*dIII
HFQR5	TGCGGATCCGATTCTCTATATCATACAGTTTCG	*Bam*HI
HFQF9	AGCGTCGACCAAAATGCAGGTCACATGGT	*Sal*I
HFQR9	TACGTCGACGATCGATCACTCCCAAATTCA	*Sal*I
CBJ2	TTCGAGTTCAAGCTGGACGTT	None
RACEOP	GCTGATGGCGATGAATGAACACTG	None
RACEIP	AACACTGCGTTTGCTGGCTTTGATG	None
KKR35	GTTGGGTAACGCCAGG	None
Primers used in Real-time PCR		**Target gene**
16SF	GCTTGCTCCCTGATGTTAGC	16S rRNA
16SR	TTGCGGAAGATTCCCTACTG	16S rRNA
SIGDF	CATGCCGCTTGTCACATATC	*sigD*
SIGDR	CGAGTTCCTCTGCAATTTCC	*sigD*
CHEYF	CAGCATTTATGCGAATGATGA	*cheY*
CHEYR	CTTCAAGCACACGGTCAGC	*cheY*

† Restriction enzyme sites are underlined in the corresponding sequence.

2.7 *Real-time PCR*

PY79 and PY79Δ*hfq* strains were grown in PRBG medium and cells were harvested during stationary phase. Total RNA was isolated using RNeasy mini kit and treated with DNaseI. First-strand cDNA was synthesized using Transcriptor cDNA synthesis kit. Real-time PCR was carried out with Light Cycler SYBR Green I Master kit. PCR amplification conditions were as follows: pre-incubation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 55–60°C (depending upon the Tm of primer) for 20 s, and extension at 72°C for 30 s. All Real-time PCR experiments were performed in triplicates. Quantification was performed using the Lightcycler 480 software (Version 1.5 Roche). For each amplification run, the calculated threshold cycle (*Cp*) for each gene amplification was normalized to the *Cp* of the 16S rRNA gene, amplified from the corresponding sample before calculating the difference (fold) between the wild type and mutant, using the following formula: Fold change = $2^{-\Delta\Delta Cp}$.

2.8 *5′-RACE for determination of transcription start site (TSS)*

5′-RACE was carried out using RLM-RACE kit as per the manufacturer's instructions with few modifications. Briefly, 10 µg total RNA was treated with tobacco acid pyrophosphatase at 37°C for 1 h to convert the 5′ triphosphates to monophosphates. Reactions were stopped by phenol-chloroform extraction followed by isopropanol precipitation. Precipitated RNA was dissolved in water. This was mixed with 5′ RNA adapter (5′-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3′), heat denatured at 95°C for 5 min and then quick chilled on ice. The ligation reaction was carried out at 17°C for 8 h with T4 RNA ligase. Ligated RNA was reverse-transcribed at 50°C with Superscript III reverse transcriptase (Invitrogen) using random decamers as primers. Nested PCR was performed as per the instructions in the RLM-RACE manual. The outer RLM-RACE PCR was carried out with 5′-RACE outer primer (RACEOP) and a gene-

specific primer (CBJ2, table 2, figure 3A), while the inner RLM-RACE PCR was carried out with 5′-RACE inner primer (RACEIP) and a gene specific inner primer (HFQR1, table 2, figure 3A).

3. Results

3.1 *hfq is co-transcribed with ymaF and miaA*

It has been previously reported that the *hfq* is transcribed and that the Hfq protein is observed in both exponential and stationary phases of growth (Hammerle *et al.* 2014; Rochat *et al.* 2015). Since *hfq* is preceded by two other genes, *ymaF and miaA*, with very short intergenic sequences, it suggested that *hfq* could possibly be co-transcribed with these two genes. In order to explore whether *hfq* is co-transcribed with *ymaF* and *miaA*, RT-PCR was carried out with primers HFQF3 and HFQR1 whose sequences are complementary to the *ymaF* and *hfq* genes, respectively (figure 1A). This resulted in an amplified product of 1344 bp, thus confirming that the product comes from a single mRNA that spans the three genes and hence constitutes a single transcription unit (figure 1B, lane 4). RT-PCR with HFQF3/HFQR3 (figure 1A) and HFQF1/HFQR1 (figure 1A; figure 3A) primers whose sequences are complementary to *ymaF*/*miaA* and *miaA/hfq* further confirmed the co-transcription of *ymaF* and *miaA* with *hfq* (figure 1B, lanes 2 and 3). A control set up with RNase A treated RNA as a template for RT-PCR did not yield any PCR product (figure 1B, lane 5), thus ruling out the possibility of amplifications from genomic DNA. Hence, this study unequivocally demonstrates the presence of polycistronic *hfq* transcripts in *B. subtilis*, indicating hfq_{BS} is co-transcribed with preceding *ymaF* and *miaA* genes. This is in agreement with the data reported from a large-scale expression profiling using DNA microarray (Nicolas *et al.* 2012). The reason for the absence of polycistronic *hfq* transcripts in previous *B. subtilis* transcriptome sequencing study (Irnov *et al*. 2010; Dambach *et al*. 2013) may be that it is not expressed at the tested growth conditions.

3.2 *Promoter located upstream of ymaF shows σ^B-dependent expression*

Towards understanding the synthesis of polycistronic *hfq* transcript, we examined the DNA sequence upstream to *ymaF* ORF for the presence of putative promoters as *hfq* is found co-transcribed with *ymaF* and *miaA* genes in a single transcription unit. A putative σ^B-type promoter was identified (figure 2A) with a –35 (GCGTTTAA) and a –10 (GGGCAT) element which showed 7/8 and 5/6 matches to the consensus σ^B promoter elements –35 (RGGXTTRA) and –10 (GGGTAT), respectively, with a spacing of 16 bp (Haldenwang 1995). It was earlier reported for a few σ^B-dependent genes that a conserved 'Adenine' present at the 10th position after –10 element acts as a transcription start site (TSS). This 'A' is generally flanked by 'T' and 'G' nucleotides (Engelmann *et al*. 1995). The examination of *ymaF* promoter sequence also revealed the presence of 'Adenine' at 10th position after –10 element and was flanked by 'T' and 'G' nucleotides (figure 2A), thus further adding credence to its σ^B-type promoter.

To determine whether the putative *ymaF* promoter is indeed functional, strain 168/YF, carrying *ymaF*P-*lacZ* fusion in a plasmid pYF, was grown in LB broth and the β-galactosidase activity was determined. The determination of β-galactosidase activities during the growth revealed that the *ymaF* expression level in 168/YF is induced during stationary phase (figure 2B), thus indicating that 648 bp DNA insert in pYF carries the *ymaF* promoter. In order to determine that it is a σ^B-dependent promoter, the strains 168/YF and 1A675/YF (*sigB* mutant) were grown in LB medium and β-galactosidase activities were compared during late stationary phase (A_{600} ~4.0) as peak *ymaF* promoter activity was observed during this stage (figure 2B). In case of 168/YF the enzyme activity was 31 Miller units (MU) while 1A675/YF showed only 6 Miller units enzyme activity (figure 2C). The 5-fold decrease observed in *ymaF* promoter activity in 1A675/YF, a *sigB* mutant, thus indicated that this promoter is a σ^B-dependent promoter. The control strains 168/RB381 and 1A675/RB381 showed 2 MU enzyme activities. The data presented in this section taken together with the RT-PCR evidence of a poly-cistronic *hfq* transcript (figure 1B) and expression profiling with DNA microarray (Nicolas *et al*. 2012) strongly suggests *hfq* transcription could be regulated by σ^B. Generally, σ^B-dependent genes are known to be expressed during stress. As peak σ^B-dependent *ymaF* promoter activity is seen during late stationary phase, it suggests a possible role for Hfq during nutrient limiting conditions.

3.3 *hfq is additionally regulated by σ^H*

In an earlier transcription profiling experiment with *B. subtilis* and its *sigH* mutant, it was reported that *hfq* (earlier designated as *ymaH*) is downregulated in *sigH* mutant, suggesting regulation of *hfq* by σ^H (Britton *et al*. 2002). The identification of monocistronic *hfq* transcripts in transcriptome analysis of *B. subtilis* (Dambach *et al*. 2013) further supports this observation, although the presence of a σ^H-dependent promoter has not yet been experimentally validated. The *B. subtilis* σ^H-dependent promoters have consensus –35 (RWAGGAXXT) and –10 (HGAAT) elements separated by an optimal spacing of 14 bp (Haldenwang 1995). The putative *hfq* promoter

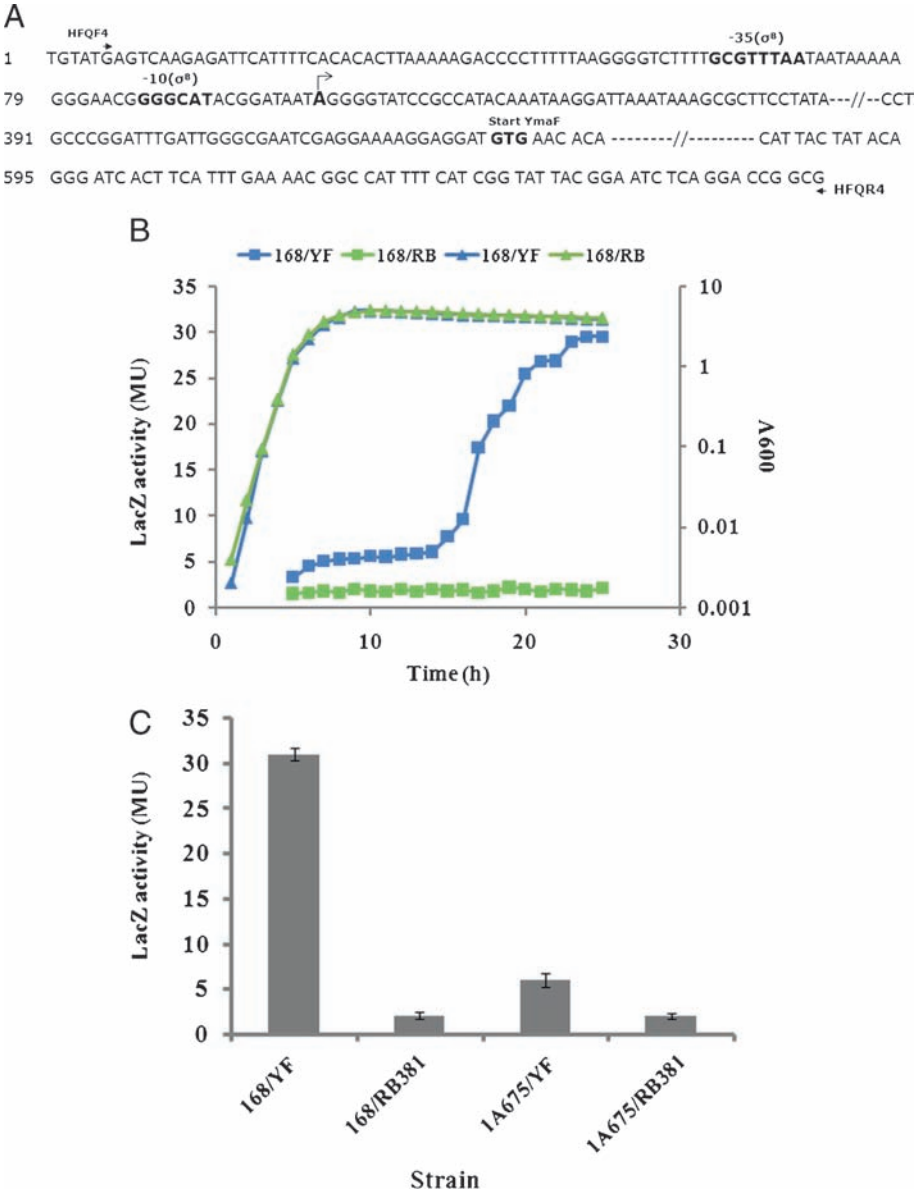

Figure 2. (**A**) The sequence of *ymaF* promoter and part of the open reading frame with positions of primers used for amplification and cloning in to pRB381. A bent arrow shown over 'A' is the putative start site for transcription. (**B**) *ymaF* promoter activity (–■–) during the growth of *B. subtilis* 168/YF (–▲–) and 168/RB (–▲–) in LB medium. Results of one representative experiment are shown out of three experiments performed under similar conditions. (**C**) *ymaF* promoter activity in *B. subtilis* 168/YF, 168/RB381, 1A675/YF and 1A675/RB381 during stationary phase. Results of one representative experiment are shown out of three experiments performed under similar conditions.

showed a –35 (GCAGGAAAA) and a –10 (CGAAA) element with 8/9 and 4/5 matches to the consensus σ^H promoter and a spacing of 13 bp between them (figure 3A). To determine if *hfq* gene is indeed regulated by σ^H, strains 168::YH and 1S20::YH, carrying *hfqP-lacZ* fusion at *amy* locus, were grown in LB broth at 37°C to early stationary phase (A_{600} ~5.0) and the β-galactosidase activities were determined. The enzyme activity was 80 MU in case of 168::YH while 1S20::YH, which has *sigH* mutated, showed half enzyme activity, indicating that the *hfq* promoter is regulated by σ^H (figure 3B). The strain 1S20::YH still showed ~40 MU enzyme activity. The reason for this could be that 1S20 is not a knockout mutant but is a point mutant and, therefore, it is possible

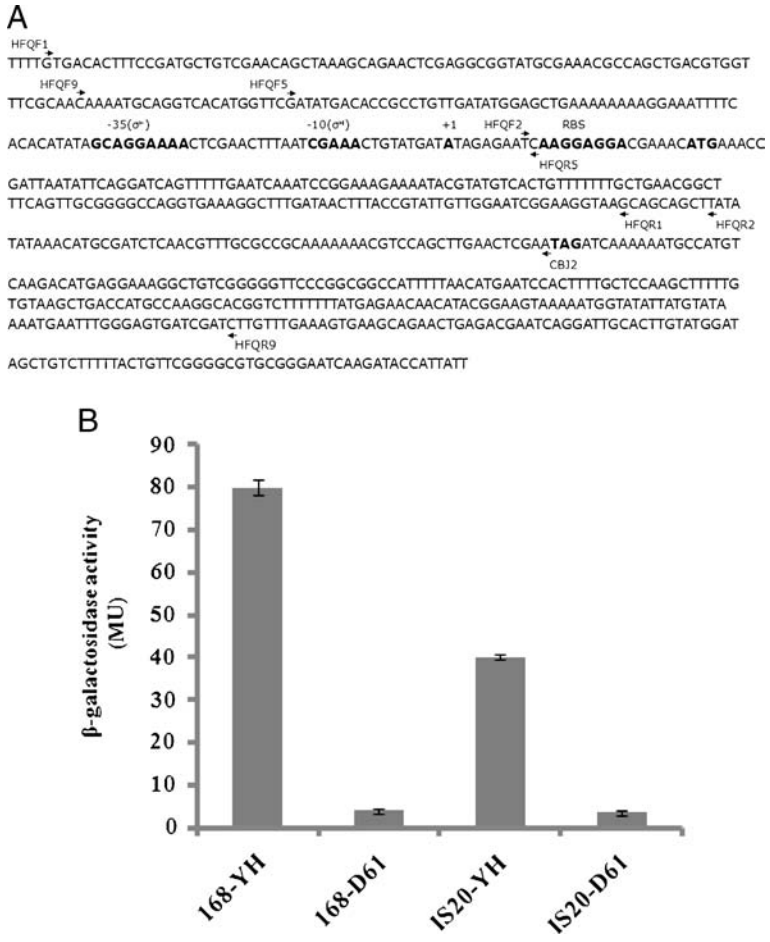

Figure 3. (**A**) The sequence of *hfq* gene in *B. subtilis*. The positions of various primers used in this study are shown by short arrows. −35 and −10 elements of σ^H-type promoter, transcription start site (+1), translational start/stop site, and RBS are shown in bold face. (**B**) *hfq* promoter activity in *B. subtilis* 168::YH, 168::DG, 1S20::YH and 1S20::DG strains. Results of one representative experiment are shown out of three experiments performed under similar conditions.

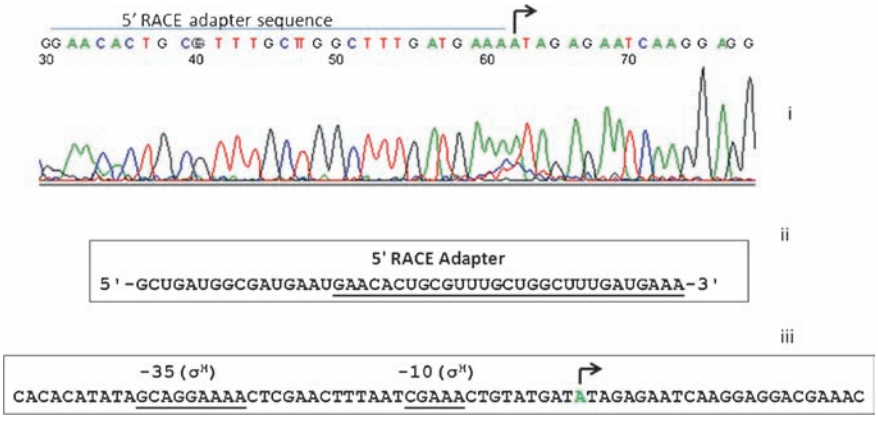

Figure 4. (**i**) Electropherogram showing sequence of the cloned 5′RACE product of *hfq* transcript. Bent arrow on 'A' denotes transcription start site. (**ii**) The sequence of 5′ RACE adapter. (**iii**) *hfq* gene sequence with σ^H-dependent promoter (underlined) and TSS (bent arrow).

that the enzyme activity observed may be due to residual activity of the mutated σ^H. The strains 168::DG and 1S20::DG showed <5 MU enzyme activity, indicating that the 103 bp insert is responsible for the transcription of β-galactosidase gene in 168::YH and 1S20::YH, thus confirming the presence of functional σ^H promoter in it.

In order to further validate the presence of a promoter present upstream to *hfq* ORF, transcription start site (TSS) of *hfq* transcript was determined by 5′-RACE. Figure 4 shows the transcription start site (TSS) of *hfq* transcript as 'Adenine' residue present at the 10th position from –10 element of the σ^H-dependent promoter. This is in line with the previously detected transcription start sites of other σ^H-dependent genes (McQuade *et al*. 2001; Ogura *et al*. 2003). Thus, our study indicates that the σ^H-dependent *hfq* transcription would result in the synthesis of monocistronic transcripts, while σ^B-dependent transcription would result in the synthesis of polycistronic transcripts.

3.4 *Hfq is required for motility and chemotaxis in B. subtilis*

Hfq has been shown to be involved in motility in many bacteria (Sonnleitner *et al*. 2003; Ding *et al*. 2004; Sittka *et al*. 2007). In order to explore whether Hfq_{BS} is involved in motility, we studied the wild-type PY79 and its isogenic *hfq* mutant, PY79Δ*hfq* (table 1). The mutant, PY79Δ*hfq*, grown in PRBG broth, showed significant impairment in motility as compared with the wild type when observed under the microscope. While PY79 cells were highly motile, PY79Δ*hfq* cells were found remarkably sluggish in their movement. Similarly, PY79 strain almost covered the complete surface on semisolid tryptone agar plates while PY79Δ*hfq* only formed a small circular zone at the centre in 15 h (figure 5A), thus demonstrating impairment of motility in *hfq* mutant. In order to assess whether chemotaxis is affected in PY79Δ*hfq*, a chemotaxis assay was performed using capillary method (Drift and Jong 1974). The quantitative capillary assay demonstrated that PY79Δ*hfq* is impaired in chemotaxis as well; while PY79 showed 865 cells/capillary in the presence of glucose (10 mM), *hfq* mutant showed only

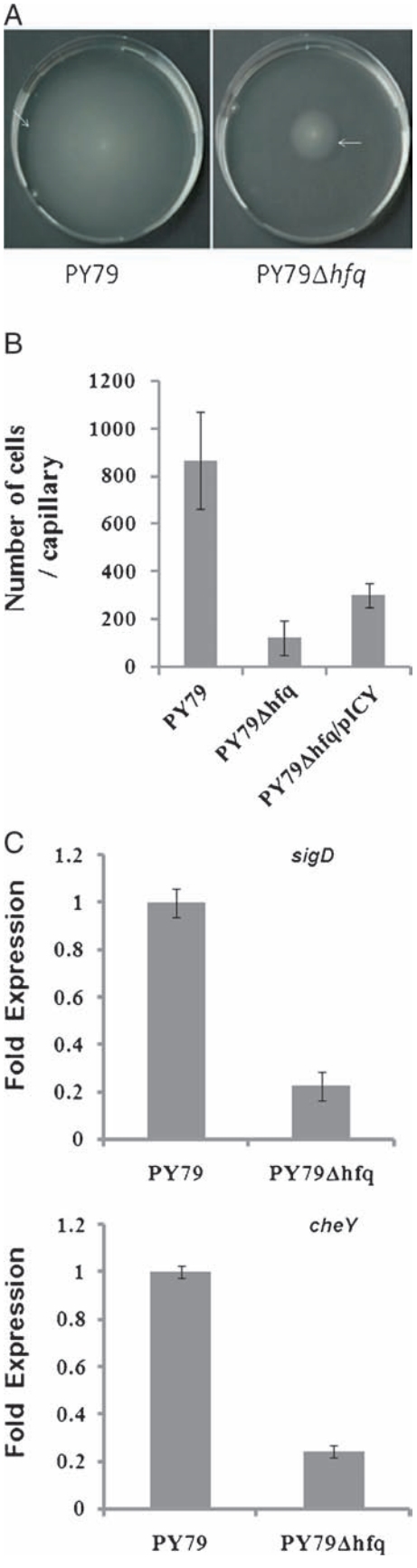

Figure 5. Hfq is required for motility and chemotaxis: (**A**) Assay for swimming motility. Arrow indicates the edge of the spreading colony. (**B**) Capillary assay for chemotaxis. The number of bacteria present in the capillary due to attractant (10mM glucose) is shown for PY79, PY79 *hfq* and PY79 *hfq*/pICY. One representative experiment is shown out of three experiments. Each assay was performed in triplicate and the numbers shown are average of three replicates. (**C**) Transcript abundance of *sigD* and *cheY* as determined by relative quantification in qRT-PCR assay. 16S rRNA transcripts were used for normalization and the data shown are mean ± standard deviation of three biological replicates.

123 cells/capillary (figure 5B). To further confirm that the impairment of chemotaxis is due to *hfq* mutation, the PY79Δ*hfq* was complemented with the intact *hfq* gene. The results of the capillary assay showed partial rescue of phenotype in PY79Δ*hfq*/pICY (300 cells/capillary; figure 5B). The reason for the partial rescue of phenotype observed in PY79Δ*hfq*/pICY may be the use of only one promoter (i.e. σ^H-dependent) in pICY for expression of *hfq*, while in wild type the *hfq* is expressed by a σ^B-dependent promoter preceding *ymaF* gene in addition to its own σ^H-promoter. Nonetheless, this study demonstrated that Hfq_{BS} is required for chemotaxis and motility.

3.5 *Hfq positively regulates the chemotaxis and motility genes*

In *Vibrio cholerae* and *Salmonella typhimurium*, it was observed that many genes required for flagellum synthesis and chemotaxis are downregulated in *hfq* mutant (Ding *et al*. 2004; Sittka *et al*. 2007). The downregulation of these genes may be responsible for the lowered motility in these strains and the same may be true in *B. subtilis*. To confirm this, qRT-PCR was carried out for quantifying the transcripts of *sigD and cheY* genes, which are part of *fla/che* operon. This operon encodes for approximately 30 genes that are required for flagellum synthesis and chemotaxis. Both *sigD* and *cheY* were found to be downregulated by ~4-fold in PY79Δ*hfq* as compared with the PY79 (figure 5C). The downregulation of *sigD* and *cheY* thus suggests downregulation of all *fla/che* genes in *hfq* mutant. Thus, like *V. cholerae* and *S. typhimurium,* genes essential for flagellum synthesis and chemotaxis are even downregulated in *B. subtilis hfq* mutant, suggesting that Hfq positively regulates the expression of these genes. The positive regulation of flagellar and chemotactic genes by Hfq in *V. cholera, S. typhimurium* and *B. subtilis* thus explains a role of this regulator in chemotaxis and motility.

4. Discussion

Several attempts have been made for the identification of small RNAs and Hfq function(s) in *B. subtilis* (Silvaggi *et al*. 2005; Hammerle *et al.* 2014; Rochat *et al.* 2015); however, little information is available about the regulation of *hfq* in this bacterium. High-throughput sequencing of *B. subtilis* transcriptome revealed the presence of only monocistronic *hfq* transcripts (Irnov *et al*. 2010; Dambach *et al*. 2013); on the other hand, a large-scale expression profiling using DNA microarray suggested the presence of polycistronic *hfq* transcripts with upstream *ymaF* and *miaA* (Nicolas *et al*. 2012). However, the latter is not yet validated with an authentic technique such as reverse-transcription PCR. The presence of a monocistronic *hfq* transcript does suggests the presence of a promoter just upstream to the *hfq* ORF, while polycistronic *hfq* transcripts with *ymaF* and *miaA* suggest the transcription of these three genes by a promoter preceding the *ymaF* ORF. In order to clear the ambiguity over the presence of polycistronic *hfq* transcripts, we carried out reverse-transcription PCR and demonstrated that *hfq* is indeed transcribed in a single transcription unit with *ymaF* and *miaA* (figure 1B). This suggests that *hfq* is transcribed as both monocistronic and polycistronic *hfq* transcripts; however, the latter may only be expressed at a particular stage of growth or during specific conditions.

As *hfq* is co-transcribed with the preceding *ymaF* and *miaA* genes (figure 1B), it is apparent that this gene is regulated by a promoter preceding the *ymaF* gene. *ymaF* expression, as determined by translational fusion with beta-galactosidase gene in *B. subtilis* 168, revealed that it is expressed during the stationary phase (figure 2B). On the other hand, *ymaF* expression in 1A675/YF, a *sigB* mutant, was significantly reduced, thus indicating that *ymaF* is regulated by σ^B (figure 2C). In *B. subtilis*, σ^B-dependent genes are generally expressed during stress. Induction of σ^B-dependent genes is also observed during stationary phase as bacteria experience stress due to nutrient limitation (Benson and Haldenwang 1992). Therefore, the expression of *ymaF* during stationary phase thus suggests its possible role under nutrient-limiting conditions. Recent reports of Hfq_{BS} providing long-term survival advantage to *B. subtilis* cells further support this view (Hammerle *et al*. 2014; Rochat *et al.* 2015).

A putative σ^H-dependent promoter was identified upstream to the *hfq* ORF, suggesting direct regulation of *hfq* by σ^H (Britton *et al*. 2002). However, this was not yet experimentally validated. Measurement of *hfq* promoter activity in *B. subtilis* 168 (wt) and 1S20, a *sigH* mutant, by generating promoter-*lacZ* reporter strains showed 50% reduction in *hfq* promoter activity in the mutant, thus demonstrating that *hfq* is indeed regulated by an σ^H-type promoter that precedes the *hfq* ORF. σ^H-dependent genes are strongly expressed during post-exponential to early stationary phase (Britton *et al*. 2002). The hfq_{BS} promoter activity was also found to be maximum during this phase.

This study, therefore, indicates that σ^H-dependent expression would result in the synthesis of monocistronic *hfq* transcripts while σ^B-dependent expression would result in the synthesis of polycistronic *hfq* mRNA. The peak σ^H-dependent expression is generally observed during early stationary phase while σ^B-dependent expression of hfq_{BS} was observed during late stationary phase (figure 2B). Hence, this study explains that the monocistronic *hfq* transcripts observed in the transcriptome sequencing of *B. subtilis* (Irnov *et al*. 2010) were originated from σ^H-dependent promoter and probably, at that stage, σ^B-dependent transcription of polycistronic *hfq* mRNA was not induced.

Regulation of *hfq* by σ^H and σ^B indicate a possible role for Hfq_{BS} during post-exponential and stationary phases at which these sigma factors are active. Post-exponential phase in *B. subtilis* is characterized by events like surfactin production, competence development, chemotaxis, motility and initiation of sporulation, etc. Recently it was observed that several genes of ComK regulon were downregulated in *hfq*$^-$ background (Hammerle *et al*. 2014). Hence, a role for Hfq in any of these processes cannot be ruled out. Regulation of *hfq$_{BS}$* by stress responsive sigma factor, σ^B, emphasizes its possible involvement in the stress response and its expression during stationary phase testifies the same.

The growth of PY79 *hfq* in PRBG medium revealed its severe impairment in motility and chemotaxis as compared with the wild type (figure 5A and B). Hfq has been reported to be required for motility in *V. cholerae, S. typhimurium* and *P. aeruginosa* (Sonnleitner *et al*. 2003; Ding *et al*. 2004; Sittka *et al*. 2007). These bacteria showed reduced motility in *hfq*$^-$ background. Further, it was observed that downregulation of flagellum and chemotaxis genes is responsible for reduced motility in *V. cholerae* and *S. typhimurium* (Ding *et al*. 2004; Sittka *et al*. 2007). In *B. subtilis*, *fla/che* gene expression has similar kinds of control as that in enteric bacteria. The organization and pattern of expression of motility genes in *B. subtilis* roughly correspond in structure to the entire class II and class III genes of enteric bacteria (Ordal *et al*. 1993). Like *V. cholerae* and *S. typhimurium,* genes encoding for flagellum synthesis and chemotactic proteins are repressed in *B. subtilis hfq* mutant as well (figure 5C). This suggests that Hfq positively regulates the expression of *fla/che* genes and thus contributes to the chemotaxis and motility.

The σ^B-dependent expression of *hfq$_{BS}$* was observed during late stationary phase. The late stationary phase is witnessed with severe nutrient scarcity. Therefore, the σ^B-dependent *hfq*$_{BS}$ expression during late stationary phase suggests its possible role during nutrient-limiting conditions. The role of Hfq to positively regulate the expression of genes involved in flagellum synthesis and chemotaxis seems to help bacteria reach out to new sources of nutrients in the already nutrient-deprived environment. The exact mechanism as to how Hfq regulate the flagellum synthesis and chemotaxis genes is not known. It would be interesting to find out that how Hfq regulates the chemotaxis and motility genes in *B. subtilis* and other bacteria (Ding *et al*. 2004; Sittka *et al*. 2007). Taken together, our results indicate an additional role for Hfq_{BS} in chemotaxis and motility.

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