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The *Acinetobacter baylyi hfq* Gene Encodes a Large Protein with an Unusual C Terminus▽

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In gammaproteobacteria the Hfq protein shows a great variation in size, especially in its C-terminal part. Extremely large Hfq proteins consisting of almost 200 amino acid residues and more are found within the gammaproteobacterial family *Moraxellaceae*. The difference in size compared to other Hfq proteins is due to a glycine-rich domain near the C-terminal end of the protein. *Acinetobacter baylyi*, a nonpathogenic soil bacterium and member of the *Moraxellaceae* encodes a large 174-amino-acid Hfq homologue containing the unique and repetitive amino acid pattern GGGFGGQ within the glycine-rich domain. Despite the presence of the C-terminal extension, *A. baylyi* Hfq complemented an *Escherichia coli hfq* mutant in vivo. By using polyclonal anti-Hfq antibodies, we detected the large *A. baylyi* Hfq that corresponds to its annotated size indicating the expression and stability of the full protein. Deletion of the complete *A. baylyi hfq* open reading frame resulted in severe reduction of growth. In addition, a deletion or overexpression of Hfq was accompanied by the loss of cell chain assembly. The glycine-rich domain was not responsible for growth and cell phenotypes. *hfq* gene localization in *A. baylyi* is strictly conserved within the *mutL-miaA-hfq* operon, and we show that *hfq* expression starts within the preceding *miaA* gene or further upstream.

The bacterial Hfq protein was originally discovered as a necessary host factor for replication of the Qβ phage RNA plus-strand in *Escherichia coli* by melting its 3′ end and permitting accessibility of phage Qβ replicase (10). Deletion of *hfq* by insertion of an omega interposon into the *E. coli* genome leads to pleiotropic phenotypes, depending on the insertion site of the interposon (35). Hfq is now regarded as global RNA chaperone involved in posttranscriptional regulation, e.g., by stimulation or protection of mRNA decay and translation or promotion of duplex formation between small regulatory RNAs and their target mRNAs by unfolding of both molecules (3, 12, 41). During the process of mRNA degradation a combination of endonucleolytic cleavages catalyzed by RNase E or III followed by exonucleolytic cleavage through polynucleotide phosphorylase and RNase II occur. Hfq-mediated protection against mRNA degradation appears if Hfq, for example, binds to the poly(A) tail of *rpsO* mRNA, encoding for the S15 protein of the 30S ribosomal subunit, which leads to decreased sensitivity for polynucleotide phosphorylase and RNase II, respectively (9). In addition, the process of translation protects mRNAs from degradation, e.g., 30S ribosome binding to the 5′-untranslated region of *ompA* mRNA protects against RNase E recognition. However, in the presence of Hfq, *ompA* mRNA translation is repressed, and mRNA decay is stimulated because Hfq interferes with ribosome binding (41). Hfq-mediated duplex formation between a small RNA (sRNA) and a target mRNA was observed, e.g., for the Hfq binding sRNAs SgrS and RyhB. Both of these sRNAs lead to translation inhibition and RNase E-dependent degradation of their mRNA targets *ptsG* (SgrS) and *sodB* (RyhB), respectively (18, 20). Conducted BLAST searches of microbial genomes highlight Hfq as a conserved protein, Hfq homologues can be found in at least half of bacterial genomes (32). The N termini of Hfq proteins especially share a high similarity with the Sm1 motif of eukaryotic Sm proteins, which bind numerous RNAs as heteroheptamers (21, 27). Sm1 is connected to a second motif called Sm2 by a region of variable length and sequence in eukaryotic cells and forms a protein structure called Sm fold consisting of an α-helix, followed by a strongly bent five-stranded β-sheet that is responsible for RNA binding and protein-protein interaction (17, 36). Crystals of the C-terminally truncated Hfq protein from *E. coli* or the full-length Hfq protein from *Staphylococcus aureus* revealed indeed a homohexameric ring-shaped structure. Despite Sm2 lacking sequence homology to eukaryotic Sm proteins, both proteins adopt the same Sm-fold structure (26, 27). Cocrystallization of *S. aureus* Hfq with a hepta-oligoribonucleotide (AU_5G) revealed that RNA binding is located within the Sm motif especially in β-sheets 2 to 5 (27). Based on this result, the assumption was made that the Hfq C terminus is not necessary for sRNA-mRNA interaction. In fact, a C-terminal 37-amino-acid truncated *E. coli* Hfq protein is able to bind sRNAs but is defective in mRNA binding (40). Interestingly, the C termini of Hfq proteins from different bacteria vary considerably in length and sequence. The longest C-terminal extensions are found in beta- and gammaproteobacteria. However, it is not known what the function of the Hfq C terminus is and why it is extremely elongated in a few organisms.

In the present study, we describe the *Acinetobacter baylyi hfq* gene and its encoded protein, which is almost twice the size of other gammaproteobacterial Hfqs due to an elongated C terminus (Fig. 1 and 2). *A. baylyi* is a gram-negative, nonpathogenic, and strictly aerobic gammaproteobacterium whose ge-

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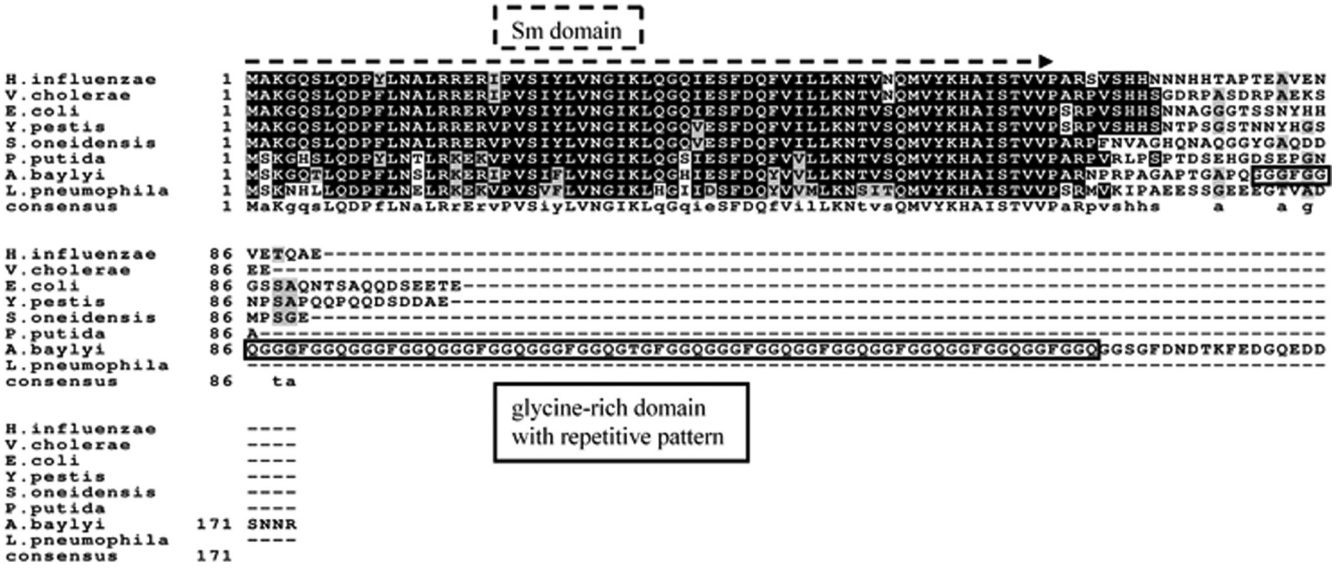


FIG. 1. Multiple sequence alignment of the *A. baylyi* Hfq amino acid sequence with seven gammaproteobacterial Hfq homologues by CLUSTAL W and visualized with BoxShade 3.2. Capital letters in the consensus sequence indicate conserved amino acids appearing in all aligned sequences. Lowercase letters indicate conserved amino acids appearing in at least two sequences. Numbers indicate the amino acid positions. Amino acids shaded gray have the same polarity as the black-shaded ones, whereas amino acids with a white background differ in their polarity from the black- and gray-shaded amino acids. The Sm domain is marked by a dotted arrow and is encoded by the first 64 amino acids in all compared sequences. The repetitive glycine-rich amino acid pattern of the *A. baylyi* Hfq sequence starts at amino acid 80 (glycine), ends at amino acid 152 (glutamine), and is outlined by a black box.

nome is completely sequenced and annotated (2). The *A. baylyi* Hfq protein contains an unusual glycine-rich domain near its C-terminal end consisting of the repetitive amino acid patterns GGGFGGQ and GGFGGQ. *hfq* is located downstream the gene *miaA* (encoding a tRNA modification enzyme) on the same DNA strand and upstream of *surA* (encoding a protein folding enzyme) on the opposite DNA strand.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in the present study are listed in Table 1. The *E. coli* strains MC4100 AM111 *hfq*1::Ω and MC4100 AM112 *hfq*2::Ω have been described elsewhere (22) and were grown in Luria-Bertani (LB) medium supplemented with 50 μg of kanamycin/ml with aeration at 37°C. *E. coli* strain MC4100 AM111/pRK415 $hfq_{A.\ baylyi}$ was cultivated in LB medium supplemented with 12 μg of tetracycline/ml and 50 μg of kanamycin/ml. Cultivation of all *Acinetobacter* strains was carried out in minimal medium (33) with aeration at 30°C. If needed, 100 μg of spectinomycin/ml, 6 μg of tetracycline/ml, or 6 μg of kanamycin/ml was added to the medium.

Plasmid and strain construction. Inactivation of the *A. baylyi hfq* gene was done by deletion of the complete open reading frame (ORF) and replacement with the Ω-interposon of plasmid pHP45Ω designed to terminate both transcriptional and translational activities and encoding spectinomycin sensitivity (25). The DNA regions next to *A. baylyi hfq* were amplified via PCR using the primers 241, 242, 243, and 244 (Table 2) and *A. baylyi* chromosomal DNA as a template. PCR products were purified with the PCR clean-up gel extraction kit (Macherey-Nagel, Düren, Germany) and subsequently cloned by using their artificial BamHI, natural AflIII, and XhoI recognition sites into cloning vector pBSKII+ (Stratagene, Amsterdam, The Netherlands), creating plasmid pBSKII+ Δ*hfq*, and used for transformation of *E. coli* DH5α cells (13). The Ω-interposon was inserted into the created BamHI-site of pBSK+ Δ*hfq*, and finally the complete fragment Δ*hfq* Ω was cut after plasmid preparation from *E. coli* DH5α pBSK+ Δ*hfq* Ω with AflIII/XhoI and transformed into the *A. baylyi* genome using natural competence (33). The specific integration of the construct was confirmed by

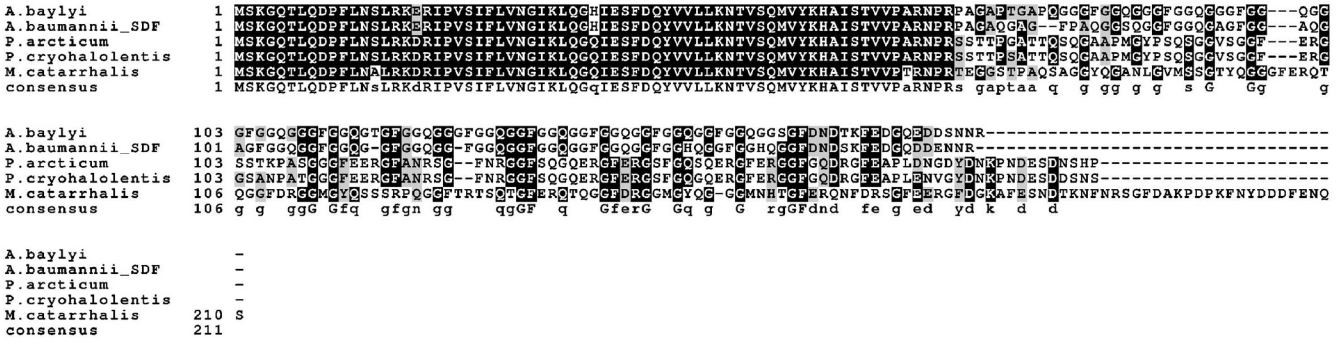


FIG. 2. Comparison of Hfq amino acid sequences from different *Moraxellaceae* by CLUSTAL W and visualized with BoxShade 3.2. Capital letters in the consensus sequence indicate conserved amino acids appearing in all aligned sequences. Lowercase letters indicate amino acids in at least two sequences. Amino acids shaded gray share the same polarity as the black-shaded ones, whereas amino acids with a white background differ in their polarity to black- and gray-shaded amino acids. Numbers indicate the amino acid position in the protein.

TABLE 1. Bacterial strains

Strain	Relevant characteristics[a]	Source or reference
A. baylyi		
ADP1	Wild type (strain BD413, ATCC 33305)	38
ADP1 Δ*hfq*	*hfq* ORF replaced by Ω-interposon; Sp^r	This study
ADP1 Δ*miaA*	Ω-interposon 55 bp downstream of *miaA* 5′ end; Sp^r	This study
ADP1 Δ*mutL*	*mutL* ORF replaced by Km-integration cassette; Km^r	6
ADP1 ΔHfq_{73aa}	Mutated *hfq* gene (chromosomal deletion of amino acids 80 to 152)	This study
ADP1/pRK415 *hfq*	Overexpression of *hfq*; Tc^r	This study
ADP1 Δ*hfq*/pRK415 *hfq*	Complementation of *hfq* deletion in *trans*; Sp^r Tc^r	This study
E. coli		
MC4100 AM111 *hfq*1::Ω	Ω-interposon inserted into *hfq* BclI site; Km^r	22, 35
MC4100 AM112 *hfq*2::Ω	Ω-interposon inserted into *hfq* KpnI site; Km^r	22, 35
MC4100 AM111/pRK415$_{A.\ baylyi\ hfq\ Plac}$	Expression of *A. baylyi hfq* controlled by its natural promoter; Km^r Tc^r	This study
MC4100 AM111/pRK415$_{A.\ baylyi\ Plac\ hfq}$	Expression of *A. baylyi hfq* controlled by the *lac* promoter of pRK415; Km^r Tc^r	This study
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	13

[a] Tc^r, tetracycline resistance; Amp^r, ampicillin resistance; Sp^r, spectinomycin resistance; Km^r, kanamycin resistance.

whole-cell PCR with the primers 241 and 244, which anneal to the insert-flanking sequences (Fig. 3 and Table 2).

Complementation of the *hfq* deletion and overproduction of Hfq in *A. baylyi* were carried out in *trans* from plasmid pRK415 (19). The *hfq* expression construct was created via primer 266 containing an EcoRI recognition site and primer 267 containing a PstI recognition site (see Table 2). The purified PCR product was cut with EcoRI and PstI and cloned into pRK415. To ensure expression from the natural *hfq* promoter and avoid additional expression from the *lac* promoter of the vector, the integration was made such that the vector promoter was downstream of the cloned *hfq* gene. Plasmid pRK415 *hfq* P*lac* was conjugated (16) with the help of plasmid pRK2013 (8) and successfully established via tetracycline resistance in *A. baylyi* Δ*hfq* and wild-type cells. Positive clones were confirmed and distinguished from *E. coli* donor cells containing plasmid pRK415 *hfq* by whole-cell PCR with the primers 4 and 5 (Table 2) annealing in the *qui-pob* operon of *A. baylyi*. The *qui-pob* operon encodes enzymes of the aromatic degradation pathway and is missing in *E. coli*. In addition, growth on *A. baylyi* minimal medium was applied because *E. coli* does not grow on this medium. The presence of the Δ*hfq* mutation was confirmed by whole-cell PCR with the primers 241 and 244.

Deletion of the 73-amino-acid repetitive glycine-rich domain in the *A. baylyi hfq* gene was achieved by amplifying two PCR products with the primer pairs 289 and 247 (289/247) and 291/246. Both DNA fragments were used as templates for an overlap extension PCR with the primer pair 247/246. The resulting *hfq* construct was missing the internal *hfq* glycine-rich domain and was transformed into *A. baylyi* Δ*hfq* cells. Positive clones expressing an $Hfq_{\Delta 73aa}$ protein were selected based on spectinomycin sensitivity. Confirmation of the clones was done by whole-cell PCR.

TABLE 2. Oligonucleotides

Primer	Sequence (5′–3′)[a]	Restriction site(s)
4	ATCAACAACGCACCACT	
5	ACCCAAGTCAAGCTGAT	
241	AGGAAACATTGGCACAATTTCAAAC	
242	CAT**GGATCC**CTTTAGACATTTTATAACTCC	BamHI
243	CAT**GGATCC**CAATCGTTAATCCTAAAACC	BamHI
244	ACGAATAAGTTCACAGTTGCTGCAA	
266	GGA**GAATTC**AAGCTTTGTAGATCCTGTTGC GGGAG	EcoRI, HindIII
267	GGA**CTGCAG**TTGCAGAGTTACCTTCTGAAC	PstI
289	GGTGGTTCAGGCTTTGACAACGATACTAA ATTTGA	
247	TAGTGAACCATTCCAGACTCAGTTTGG	
291	AAAGCCTGAACCACCTTGTGGAGCACCAG TAGGCG	
246	CAGGCTCAAGAAGACTTGCGAAACTC	
342	CTACTGCAAGCGGGAA**GGATCC**TTTGGCGT	BamHI
344	ACGCCAAA**GGATCC**TTCCCGCTTGCAGTAG	BamHI
343	CGA**CGGCCG**TTAACGATTGTTAGAATCGTC	
301	TCAAAAGATTCAATGTGGCCTTG	

[a] Restriction sites are indicated in boldface.

Deletion of *miaA* was performed by insertion of the Ω-interposon near the 5′ end of the ORF. To this end, a BamHI recognition site had to be established in the *A. baylyi miaA* gene. Two DNA fragments were created by using the primer pairs 342/343 and 344/241 by PCR. Both products were included as a template in an overlap extension PCR leading to the insertion of a BamHI site 52 bases downstream of the *miaA* 5′ end without interruption of the reading frame. This construct was amplified with the primer pair 241/343, cut with SalI/EagI, cloned in pBSK+II, and established in *E. coli* cells. Positive clones were selected via ampicillin resistance and blue-white screening. The Ω-interposon was cut out of pHP45Ω with BamHI and ligated with plasmid pBSK+ *miaA* cut with the same enzyme. Positive clones were selected by ampicillin and spectinomycin resistance, and the complete *miaA* deletion construct was cut by PvuI/SalI (both are natural enzyme recognition sites of the *A. baylyi hfq* genomic region), gel purified, and transformed into *A. baylyi* wild-type cells. Positive clones were selected based on spectinomycin resistance and confirmed by whole-cell PCR.

Complementation of an *hfq* deletion in *E. coli* Δ*hfq* strains by *A. baylyi hfq* was fulfilled by transfer of plasmid pRK415 $hfq_{A.\ baylyi}$ into *E. coli* MC4100 strain AM111 *hfq*1::Ω and screening for positive clones by tetracycline and kanamycin resistance. Cloning of *hfq* with the HindIII recognition site of primer 266 resulted in a plasmid expressing *hfq* from the pRK415 *lac* promoter. This plasmid was also established in *E. coli* MC4100 strain AM111 *hfq*1::Ω.

RNA isolation and Northern blot analysis. Total RNA was isolated by a procedure described previously (24). The RNA quality and concentration were determined from the ratio of the optical density at 260 nm (OD_{280}) to that at 280 nm. Then, 15 μg of purified RNA was heat denatured for 5 min at 65°C and separated on a 1.2% formaldehyde-agarose gel in 1× RNA loading dye (1 ml of 5× RNA loading dye was composed of 8 μl of 500 mM EDTA, 200 μl of 100% glycerol, 72 μl of 37% [vol/vol] formaldehyde, 308 μl of formamide, 400 μl of 10× running buffer [200 mM morpholinepropanesulfonic acid, 50 mM sodium acetate, 5 mM EDTA; pH 7], 2 μl of saturated bromophenol blue, and 10 μl of deionized water) and transferred to a Hybond-N+ nylon membrane (Amersham

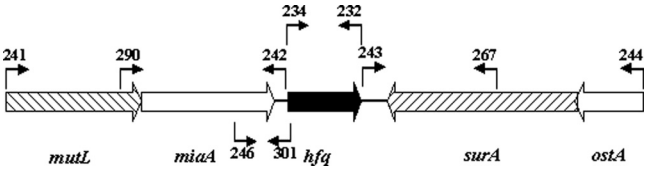


FIG. 3. Schematic representation of the genomic localization of *A. baylyi hfq* and primer sites for *hfq* manipulation, MiaA, Hfq, and SurA Northern blot detection and *hfq* RT-PCR. Primer sites are indicated by arrows and numbers (see Table 2).

Biosciences, Freiburg, Germany) by capillary blotting in 10× SSC (1.5 M sodium chloride, 0.15 mM sodium citrate) overnight. The membrane was dried at room temperature, and the RNA was covalently bound by a UV cross-linker (Amersham Biosciences) for 90 s at 1,200 μJ/cm^2. Specific RNA detection was performed by the digoxigenin (DIG) labeling and detection system (Roche Applied Sciences, Mannheim, Germany).

Western blot analysis. To obtain *A. baylyi* total protein crude extract, 1 ml of cells was treated for 15 min on ice with 25 μl of 2% deoxycholic acid. Afterward, the total protein was precipitated with 30 μl of 40% trichloroacetic acid, centrifuged 5 min at 14,000 × *g*, and dissolved in deionized water. Separation of total protein was carried out with a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel, and the protein concentration was measured by using a Bradford assay. Then, 20 μg of the total protein was applied per lane, after dilution with 1 volume of SDS loading dye (12% SDS [wt/vol], 6% β-mercaptoethanol [vol/vol], 30% glycerol [vol/vol], 0.05% Coomassie brilliant blue G-250 [wt/vol], 150 mM Tris-HCl; pH 7) and incubation at 65°C for 5 min before loading. After separation, the protein was transferred onto a nitrocellulose membrane by using an electroblotter (Serva Electrophoresis, Heidelberg, Germany) at 200 mA for 2 h. The membrane was blocked in 5% skim milk powder for 1 h at room temperature before incubation with anti-Hfq antibodies (raised against *Pseudomonas putida* Hfq and kindly provided by Gerhard Burchardt, University of Greifswald, Greifswald, Germany) diluted 1:1,000 for 1 h at room temperature. Detection of specific Hfq–anti-Hfq interaction was performed with a Vectastain ABC kit (Vector Laboratories, Peterborough, England).

RT-PCR analysis. RNA of *A. baylyi* grown until early stationary phase was isolated and treated with DNase I (Roche Applied Sciences) to remove any DNA contamination. Then, 1 μg of DNA-free RNA was denatured at 70°C for 5 min together with 2 pmol of primer 301, which is complementary to *A. baylyi hfq* and anneals 119 bases downstream of the *hfq* 5′ end. Reverse transcription (RT) was performed with 100 U of Moloney murine leukemia virus reverse transcriptase RNase Minus Point Mutant (Promega, Mannheim, Germany) in a total volume of 25 μl at 45°C for 1 h. Finally, 1 μl of the created cDNA was used as a template in a PCR with the primers 301 and 246 (see Fig. 3 and Table 2). As controls, the reaction was separately done without the addition of reverse transcriptase on the one hand (a negative control for the RT reaction) and *A. baylyi* chromosomal DNA as cDNA template on the other hand (a positive control for the PCR).

RESULTS

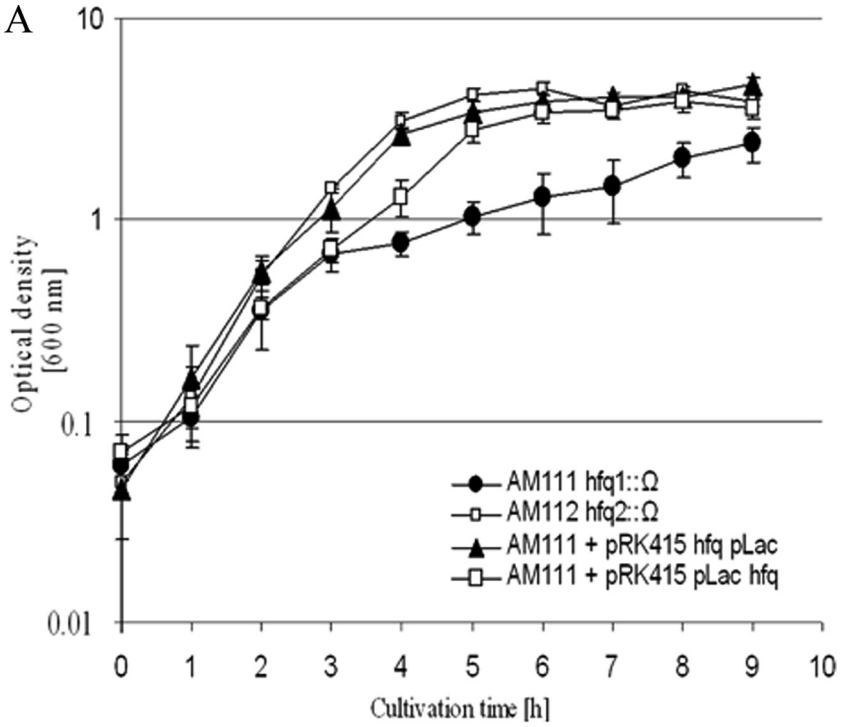
In silico identification of an unusually large Hfq protein encoded by *A. baylyi*. *A. baylyi* strain ADP1 encodes an unusually large Hfq protein (174 amino acids) with highly significant homology between amino acid residues 1 and 66 to those of other gammaproteobacterial Hfqs, including the Hfq Sm motifs (Fig. 1). In contrast, the C-terminal end of *A. baylyi* Hfq is more than three times larger and contains a hydrophobic glycine-rich domain. Detailed BLAST searches identified this domain as special Hfq feature within the *Moraxellaceae* family. Whereas Hfq proteins of the genus *Acinetobacter* show the strictly repetitive amino acid patterns GGGFGGQ and GG FGGQ (starting at positions 81 and 126 of *A. baylyi* Hfq [Fig. 1]), Hfq homologues of the genera *Psychrobacter* and *Moraxella* contain no obvious patterns compared to *A. baylyi* Hfq within their glycine-rich Hfq domains (Fig. 2, consensus line). A PHI-BLAST (pattern hit initiated) search revealed GGGFGGQ also within DNAJ/DNAJ-like chaperones of different bacteria, which contain a glycine-rich domain and are involved in protein folding, protein transport, and response to cell stress (42). We also observed this pattern in DNA-binding proteins, e.g., in DNA polymerase III from *Frankia* sp.

***A. baylyi* Hfq complements an *E. coli hfq* deletion.** *A. baylyi* Hfq functionality was investigated by complementation of growth and cell phenotype effects of *E. coli* Δ*hfq* strains. *A. baylyi* Hfq was expressed in *trans* and controlled by the natural *hfq* promoter (pRK415 *hfq*) or by the *lac* promoter (pRK415 P*lac hfq*), respectively. Since *A. baylyi* does not contain a *lac* operon, the use of IPTG (isopropyl-β-D-thiogalactopyranoside) was not necessary. We know from previous work that this promoter functions in *A. baylyi*. Both plasmids were transformed into the *E. coli* MC4100 strain AM111 *hfq*1::Ω, which possesses an Ω-interposon in the *hfq* BclI recognition site (117 bases downstream of the *hfq* start codon) and shows effects on growth and cell size. Strain *E. coli* MC4100 AM112 *hfq*2::Ω contained the Ω-interposon at the *hfq* KpnI site (232 bases downstream of the *hfq* start codon) and showed no phenotype (35). The latter strain was used as a reference strain in the present study. Although restoring normal growth by complementation of strain AM111 *hfq*1::Ω with the *E. coli hfq* gene was not possible (35), strains expressing *A. baylyi* Hfq instead showed the same growth behavior and cell size like the reference strain in LB complex medium (Fig. 4). Hfq controlled by its own promoter and expressed from the low-copy-number plasmid pRK415 (a derivate of RK2) is sufficient to restore the original growth behavior, whereas additional expression from the *lac* promoter results in a slightly retarded growth and elongated cells compared to the reference strain. These observations indicate that the concentration of Hfq must be well balanced to perform its natural function. We never observed biphasic growth behavior of AM111 *hfq*1::Ω complemented with *A. baylyi hfq*. However, strain AM111 *hfq*1::Ω showed a biphasic growth in LB medium supplemented with 171 mM sodium chloride, which has been described before (35). This biphasic growth behavior was characterized as salt-dependent and not observed in LB medium supplemented with 0, 0.085, or 0.5 M sodium chloride (35).

Growth effects of *hfq* manipulation in *A. baylyi*. The deletion of *A. baylyi hfq* was done by replacing the complete ORF with an Ω-interposon and complemented by expression of *hfq in trans* from the low-copy plasmid pRK415 or by replacing the Ω-interposon with a partially deleted (Δ73 amino acid [Δ73aa] = glycine-rich domain) *A. baylyi hfq* gene. Overexpression of Hfq was carried out by transfer of the plasmid pRK415 *hfq* into *A. baylyi* wild-type cells (Fig. 5). The deletion of *hfq* led to a strong decrease in the growth rate (*A. baylyi* wild type, $\mu = 0.92\ h^{-1}$; *A. baylyi* Δ*hfq*, $\mu = 0.12\ h^{-1}$) and a lower final OD of the culture than in wild-type cells. Restoring the original growth behavior with *hfq* expression in *trans* from the low-copy replicating plasmid pRK415 and controlled by its own promoter was not completely possible and resulted in a strain starting exponential growth after a long lag phase and reaching a lower final OD. Establishing pRK415 without *hfq* in *A. baylyi* had no effect on the growth rate, but cultures reached a higher final OD compared to wild-type cells ($OD_{600} = 1.7$ for wild-type cells and $OD_{600} = 2.7$ for pRK415-containing cells [data not shown]). In contrast, chromosomal complementation of the deletion with a smaller *A. baylyi hfq* gene resulted in restoring the original growth behavior of *A. baylyi*, indicating that the C-terminal part, which is deleted in this strain (Δ73aa) is not required for normal growth. Transfer of an *hfq* expressing plasmid controlled by the *lac* promoter into *A. baylyi hfq* mutant cells was not successful, perhaps due to the constitutive *hfq* expression, which led to a toxic Hfq accumulation. Interestingly, overexpression of Hfq regulated by its own promoter in addition to the chromosomal wild-type *hfq* allele led to the same growth rate but to a higher final OD of the culture.

Furthermore, we included a Flag tag containing two stop

A



B

Strain	Ø Cell size [µm]	Growth rate µ [h^{-1}]
AM111 *hfq*1::Ω	3.63 ± 0.07	1 = 1.03 ± 0.07 2 = 0.37 ± 0,05
AM112 *hfq*2::Ω	2.68 ± 0.19	1.15 ± 0.17
AM111 pRK415 *hfq Plac*	2.57 ± 0.21	1.15 ± 0.23
AM111 pRK415 *Plac hfq*	3.02 ± 0.44	0.94 ± 0.14

FIG. 4. Growth comparison of *E. coli hfq* mutants with or without a plasmid expressing *A. baylyi* Hfq. (A) Growth curves of cells grown in LB medium supplemented with 170 mM NaCl and antibiotics. Strains were grown overnight, diluted 1/100, and incubated at 37°C with aeration, and growth was monitored by measuring the absorbance at 600 nm every hour. (B) Cell size and growth rate comparison of *E. coli hfq* mutants with or without a plasmid expressing *A. baylyi* Hfq. Thirty cells collected from cultures were measured at an OD_{600} of 1. For *E. coli* MC4100 AM111 *hfq*1::Ω showing a biphasic growth, the growth rates were determined at OD_{600} of 0.4 (growth rate 1) and an OD_{600} of 0.8 (growth rate 2).

codons at different Hfq positions, respectively, by a modified method of a one-step gene inactivation epitope tagging of chromosomal genes (37) in the *A. baylyi hfq* gene. A mutant with the tags inside the Sm domain showed similar growth defects compared to the total *hfq* deletion, whereas an insertion of the tag before the *hfq* stop codon resulted in a very weak effect on growth compared to wild-type cells (data not shown). This indicates that *hfq* and especially the N-terminal protein part is necessary for normal growth in *A. baylyi*.

Verification of an unusually large Hfq in *A. baylyi*. Given the observation that the unusual C-terminal extension of *A. baylyi* Hfq was not necessary for normal growth, we investigated whether the whole ORF was expressed. Therefore, all *A. baylyi hfq* strains were analyzed with regard to Hfq mRNA and protein expression (Fig. 6). Northern blot analysis with *hfq*-specific PCR probes revealed that in fact *hfq* deletion and complementation were successful on the mRNA level. In the wild type, a 525-base Hfq mRNA was expected, whereas deletion of the *hfq* ORF or of the glycine-rich domain should result in the absence of Hfq mRNA or in a 306-base mRNA, respectively. Indeed, the *A. baylyi* Δ*hfq* strain expressed no *hfq* mRNA any longer (Fig. 6C). Chromosomal *hfq* complementation resulted in a smaller *hfq* mRNA due to the insertion of the *hfq* gene without its glycine-rich domain (Δ219 bp) compared to the wild-type mRNA. As expected, the *hfq* mRNA level of the wild type and the chromosomally complemented Δ*hfq* strain appeared to be similar. In contrast, the in *trans* complementation by overexpression from the low-copy-number plasmid pRK415 led to a much higher amount of *hfq* mRNA (Fig. 6C). This observation strengthens the earlier assumption that Hfq is only effective at a certain concentration.

Correspondingly, the Hfq protein expression was investi-

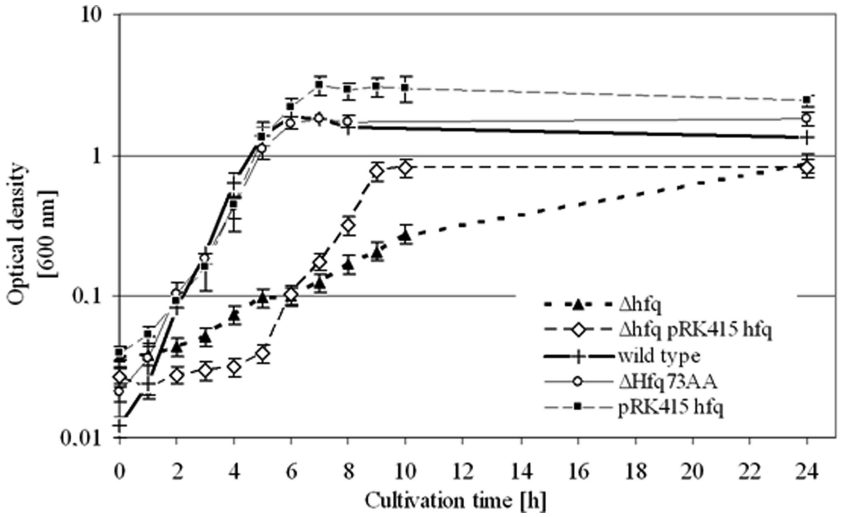


FIG. 5. Growth of *A. baylyi* strains manipulated in the *mutL-miaA-hfq* operon in minimal medium supplemented with 10 mM succinic acid and antibiotics. Strains were grown overnight, diluted 1/25, and incubated at 30°C with aeration, and the growth was monitored by measuring absorbance at 600 nm every hour.

gated in all *hfq* mutant *A. baylyi* strains by Western blot analysis with specific anti-Hfq antibodies raised against *P. putida* Hfq (Fig. 6). Both proteins share an identity of 83% in the N-terminal part (amino acid residues 1 to 70). *A. baylyi* Hfq could be detected as a 17-kDa band, confirming the existence of an unusually long Hfq in *A. baylyi* (Fig. 6A, lane WT). *A. baylyi* Hfq could also be detected as 17-kDa protein in the *hfq* complemented and the overexpressing strain (Fig. 6A, lanes *hfq*C and *hfq*P). According to the signal strength (plasmid versus chromosomally encoded *hfq*), overexpression of Hfq was successful, since the plasmid-containing strains caused a much stronger signal. In addition to the specific Hfq signal, we noticed a nonspecific binding of the antibody to a 15-kDa protein in all *A. baylyi hfq* strains. Western blot analysis of total protein from an *E. coli* strain expressing *A. baylyi hfq* did not reveal this band; it showed only one band according to Hfq of the same size as in *A. baylyi* wild type (Fig. 6B, lane $hfqP_{Eco}$). *P. putida* Hfq was detected at 10 kDa, a finding which agrees with the annotation of the protein (Fig. 6A, lane *Pfl*). The signal of the 15-kDa protein again was missing, indicating that it is derived from a nonspecific binding of the antibody with an *A. baylyi* protein.

***A. baylyi hfq* transcription starts within the *miaA* coding region or further upstream.** In contrast to the deviation from

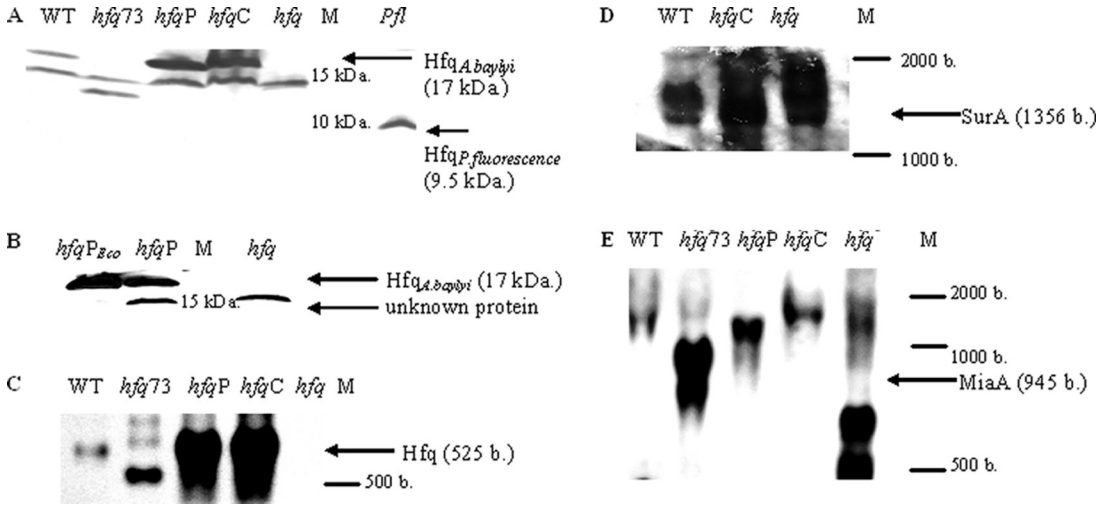


FIG. 6. Analysis of *hfq* expression in *A. baylyi hfq* strains used in the present study by Western and Northern blot experiments. (A and B) Hfq Western blot analysis of 20 μg of total protein derived from all *hfq* mutant *A. baylyi* strains using a polyclonal antibody raised against *P. putida* Hfq. (C) Northern blot analysis of *hfq* mRNA detected with a specific DIG-labeled PCR probe (primer pair 232/234) in total RNA derived from all *hfq* mutant *A. baylyi* strains. (D) Detection of *surA* transcript with a specific DIG-labeled PCR probe (primer pair 243/267). (E) Detection of *miaA* transcript using a specific DIG-labeled PCR probe (primer pair 242/290). Abbreviations: WT, ADP1 wild type; *hfq*73, ADP1 $Hfq_{\Delta 73aa}$; *hfq*P, ADP1/pRK415 *hfq*; *hfq*C, ADP1 Δ*hfq*/pRK415 *hfq*; *hfq*, ADP1 Δ*hfq*; *Pfl*, *P. fluorescence* wild type; $hfqP_{Eco}$, *E. coli*/pRK415 $hfq_{A.\ baylyi}$ *Plac*; M, PageRulerProtein ladder (Fermentas, St. Leon-Roth, Germany) or ssRNA ladder (New England Biolabs, Frankfurt am Main, Germany).

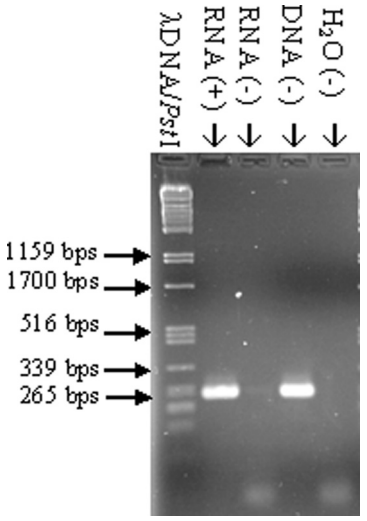


FIG. 7. RT-PCR analysis of the *A. baylyi miaA-hfq* region. Lanes: RNA (+), RT-PCR with *A. baylyi* total RNA as a template; RNA (−), RT-PCR with *A. baylyi* total RNA as a template but without reverse transcriptase; DNA (−), RT-PCR with *A. baylyi* chromosomal DNA as a template; H_2O (−), PCR with H_2O instead of DNA as a template; λDNA/PstI, λDNA cut with PstI.

other bacteria in terms of Hfq C-terminal size and sequence conservation, *hfq* genomic localization is strictly conserved among gammaproteobacterial genomes. *A. baylyi hfq*, being no exception to this, is encoded within the *mutL-miaA-hfq* operon, whose transcription has been extensively studied in *E. coli* (34). This conservation does not apply to the genes downstream of *hfq* among gammaproteobacteria. In many cases, *hfl* genes encoding enzymes for bacteriophage λ lysogeny (4) follow *hfq*. Species of the family *Moraxellaceae* encode different genes downstream of *hfq*. In *Moraxella catarrhalis* it is *kpsF* encoding for arabinose-5-phosphate isomerase (1), and in *A. baylyi* it is *surA* encoding for a peptidyl-prolyl *cis-trans* isomerase. RT-PCR analysis was performed with RNA from *A. baylyi* to determine whether *hfq* transcription starts within the *miaA* coding region or further upstream. An RT step was carried out with primer 301 (Fig. 3) annealing from bp 119 till bp 97 downstream of the *hfq* 5′ end on the negative strand, whereas the second PCR primer 246 (Fig. 3) was located from bp 35 till bp 10 upstream of the *miaA* 3′ end, respectively. The RT-PCR yielded a ~265-bp product that was missing in the control reaction without reverse transcriptase (Fig. 7), indicating that *hfq* transcription starts at least partly within the *miaA* ORF or further upstream.

Influence of *hfq* on the expression of neighboring genes. The *A. baylyi hfq* gene is located between the genes *surA* and *miaA* (Fig. 3). An *hfq* deletion could possibly affect *surA* or *miaA* expression, e.g., by destabilization of their transcripts, and subsequently the observed effect could be caused by *miaA* or s*urA* rather than by *hfq*. Both neighbor genes are involved in important processes. *miaA* expression leads to tRNA modification by transfer of an isopentenyl moiety from dimethylallyl pyrophosphate to adenosine 37 of tRNAs (5, 28), and *surA* encodes a rotamase that is involved in protein folding (14). Therefore, possible effects toward transcript stability of both mRNAs were investigated. To address this issue, we studied the influence of *hfq* loss and insertion of the Ω-interposon in *miaA* and *surA* (Fig. 6D and E) via Northern blot analysis with specific DNA probes (*miaA* primer pair 290/242, *surA* primer pair 267/243). *surA* and *miaA* mRNA were detected in total RNA isolated from cells from the stationary growth phase. The deletion of *hfq* or the insertion of the Ω-interposon had no influence on the mRNA level of the downstream gene *surA* (Fig. 6D). *miaA* is encoded on the same DNA strand as *hfq*, and *hfq* expression starts within or upstream of the *miaA* ORF. Northern blot experiments revealed that (except for the *A. baylyi hfq* mutant) the *miaA* mRNA was detected in the size range between 1,500 and 2,000 bases. Given the lengths of the *miaA* gene (945 bp) and the *hfq* gene (525 bp), it is likely that there is cotranscription. In addition to the *miaA* mRNA, we saw two strong abundant RNAs with sizes between 500 and 1,000 bases appearing in the *A. baylyi hfq* mutant (Fig. 6E, lane *hfq*). It is known that Hfq specifically destabilizes its own mRNA at the posttranscriptional level by binding at two positions in the 5′-untranslated region of its mRNA, which results in inhibition of the translation initiation complex (39). Together with the fact that *E. coli hfq* transcription is regulated within the *miaA* gene by two promoters whose expression results in long untranslated transcripts (891 and 488 bases), a lack of Hfq could result in the accumulation of these 5′-untranslated Hfq mRNAs (34). Our hypothesis is supported by the fact that complementation of the *hfq* mutation in *trans* leads to the loss of both small abundant *hfq* transcripts (Fig. 6E, lane *hfq*C). In addition, also in case of the *hfq* wild type, C-terminal truncated, and Hfq overexpression situations, wherever Hfq was present, both small RNAs were also not detected (Fig. 6E, lane WT, *hfq*73 and *hfq*P). We checked whether insertion of the Ω-interposon is responsible for the *miaA* mRNA instability and would lead to the observed Δ*hfq* growth defect. Therefore, we introduced the Ω-interposon into the *miaA* gene. We were concerned that a complete replacement of *miaA* with the interposon would lead to secondary effects due to *hfq* transcription failure. Along these lines was the finding that a complete deletion of *miaA* during the creation of single deletion mutants in *A. baylyi* was not possible (6). For both reasons, we created an artificial BamHI site by replacing TTGGC into CCTAG 55 bases downstream of the *miaA* start codon to create an incorporation site for the Ω-interposon. At this site the Ω-interposon was inserted to inactivate the *miaA* gene. A*. baylyi mutL* (preceding the *miaA* gene and kindly supplied by Valerie de Berardinis, Genoscope, France), *miaA* and *hfq* mutants were compared in growth experiments (Fig. 8) showing that deletion of *hfq* revealed the mutant with the biggest effect on growth. The effect of the *miaA* mutation was intermediate, whereas *mutL* mutants showed no apparent effect compared to wild-type cells.

Given that *A. baylyi* Hfq is able to influence *E. coli* cell size (35), we checked the cells of all *A. baylyi* strains that are manipulated in the *mutL-miaA-hfq* operon (Fig. 9). Interestingly, *hfq* deletion (Fig. 9G), as well as overexpression (Fig. 9D), led to the inability of the cells to assemble in chains. Complementation of the deletion in *trans* (Fig. 9H) could not restore the original cell arrangement in contrast to chromosomal complementation (Fig. 9A), indicating that the amount of Hfq is critical for the cell phenotype. An effect of the cell phenotype due to the plasmid pRK415 (Fig. 9C) can be ex-

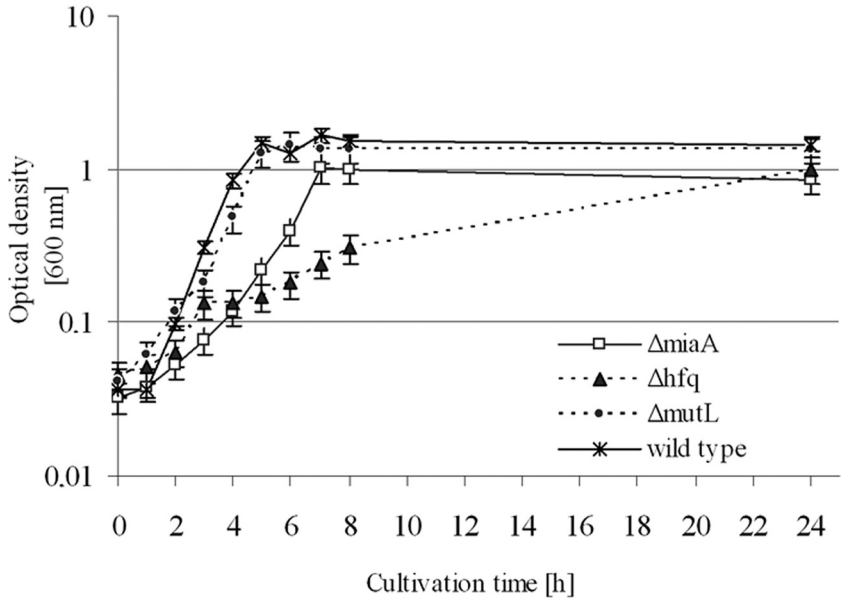


FIG. 8. Growth of *A. baylyi* strains manipulated in the *mutL-miaA-hfq* operon in minimal medium supplemented with 10 mM succinic acid and antibiotics. Strains were grown overnight, diluted 1/25, and incubated at 30°C with aeration, and the growth was monitored by measuring the absorbance at 600 nm every hour.

cluded. Neither *mutL* (Fig. 9F) nor *miaA* (Fig. 9E) mutants are affected in their cell phenotype compared to wild-type cells (Fig. 9B). Taken together, the described experiments provide evidence that the effects observed upon deletion of *hfq* are due to the absence of this gene and not to the disturbed expression of the neighboring genes.

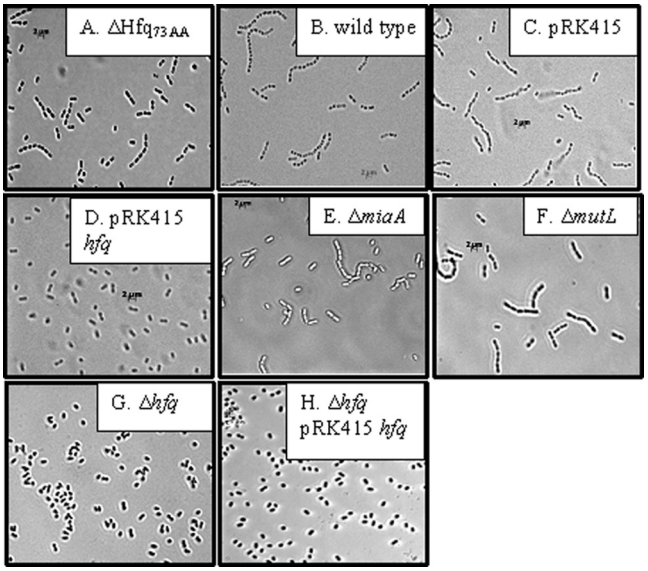


FIG. 9. Microscope comparison of different *A. baylyi* strains. The cells were grown in minimal medium supplemented with 10 mM succinic acid as carbon source and incubated at 30°C in flasks with aeration until an OD of ~1 at 600 nm was reached.

DISCUSSION

Hfq is known to show a strong variation in its C terminus in gamma- and betaproteobacteria (40). Searching redundant protein databases showed that (except for a few betaproteobacteria especially *Burkholderia* species) the largest known Hfq proteins are annotated in members of the gammaproteobacterial family *Moraxellaceae*. The annotated Hfq lengths are between 168 and 174 amino acids for *Acinetobacter* species, 183 to 203 amino acids for *Psychrobacter* species, and up to 210 amino acids for *M. catarrhalis* (1, 38). Among the *Moraxella* spp., Hfq proteins differ in terms of the length of the C-terminal domain, its sequence, and the downstream genes (either *surA* in *Acinetobacter* species or *kpsF* encoded in other *Moraxellaceae*). In *Burkholderia* genomes such as in *Burkholderia pseudomallei*, small (79-amino-acid) and large (216-amino-acid) Hfq homologues have been annotated but not yet verified. Except for the abundance of glycine residues between the N and C termini, there is no obvious sequence conservation comparing both large Hfq homologues of *Burkholderia* and *Acinetobacter* spp. Surprisingly, the small and large Hfq *Burkholderia* proteins themselves share only low sequence conservation even in the conserved N terminus (53% between amino acids 9 and 79). In addition, the genomic locus is not conserved: neither the *Burkholderia* small Hfq protein nor the large Hfq protein is encoded within the *mutL-miaA-hfq* operon. *Burkholderia* small *hfq* precedes the *hfl* genes, which act as GTPases and are responsible for high frequency of bacteriophage lambda lysogenization in *E. coli* (23). This genetic localization also seems to be common for many small *hfq* genes in gammaproteobacteria. *A. baylyi* Hfq is twice as long as most gammaproteobacterial Hfq proteins (see references 7, 29, and 31). It is encoded upstream of *surA*, a peptidyl *cis-trans*

isomerase. This enzyme class is involved in the correct folding of many eukaryotic and prokaryotic proteins (11, 15). In the present study, we could show by Northern blot analysis and RT-PCR that *hfq* transcription may start within the preceding *miaA* gene, as is the case in *E. coli* (34). All large Hfq homologues contain a glycine-rich domain between their N- and C-terminal ends, but only *Acinetobacter species* show a strictly repeated pattern within this domain. Searching the nonredundant protein databases showed that this pattern can be found in DNAJ-like chaperones (42). Until now, there have been no data available for the functionality of this protein region. For *P. putida* it was shown that its Hfq of 86 amino acids can complement *hfq* deletion effects in *E. coli* (30). We report here that the large gammaproteobacterial Hfq from *A. baylyi* (174 amino acids) shows the same ability to complement the *hfq* deletion effects on growth behavior and cell phenotype in *E. coli*. Furthermore, a complete *hfq* deletion in *A. baylyi* resulted in a drastic disruption of growth and change in the cell phenotype which could not completely be restored with the same expression plasmid used for *E. coli hfq* complementation, whereas chromosomal complementation resulted in complete restoration. The experiments reported here indicate that the amount of Hfq in the cell needs to be controlled: overexpression led to the loss of chain assembly; *in trans hfq* complementation resulted in failure to restore wild-type growth behavior. In contrast, the glycine-rich domain of *A. baylyi* Hfq is not important for normal growth and cell phenotype, as shown by the chromosomal deletion of the 73-amino-acid glycine-rich domain in *A. baylyi*. A deletion of *hfq* in *A. baylyi* has a strong effect on growth by causing an elongated lag phase but also a loss of the ability of exponential cell splitting. To exclude secondary effects on *miaA* transcript stability by the Ω-interposon, we investigated partial *miaA* deletion. Complete *miaA* disruption is not possible because it would interfere with *hfq* transcription, and we saw no effects on cell arrangement and only slight growth defects with exponentially growing cells in a strain where only 5% of the full-length of *miaA* is expressed (from the ATG to the start of the Ω-interposon).

In summary, *A. baylyi* Hfq is the second described member of large Hfq proteins encoded within the family *Moraxellaceae*. *A. baylyi* Hfq differs from *M. catarrhalis* in the amino acid composition of the large C-terminal domain and the genetic localization regarding *hfq* downstream genes. *A. baylyi* and *M. catarrhalis* share the same upstream gene (*miaA*) and the feature that *hfq* transcription starts within *miaA* or further upstream. Remarkable and unique for *Acinetobacter* is the Hfq glycine-rich domain, consisting of a strictly repeated amino acid pattern.

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