Journal of Bacteriology

Functional Characterization of the RNA Chaperone Hfq in the Opportunistic Human Pathogen Stenotrophomonas maltophilia

Emanuela Roscetto, Tiziana Angrisano, Valerio Costa, Mariassunta Casalino, Konrad U. Förstner, Cynthia M. Sharma, Pier Paolo Di Nocera and Eliana De Gregorio

J. Bacteriol. **2012, 194(21):5864. DOI: 10.1128/JB.00746-12. Published Ahead of Print 24 August 2012.**

JB
Journals.ASM.org

Functional Characterization of the RNA Chaperone Hfq in the Opportunistic Human Pathogen *Stenotrophomonas maltophilia*

Emanuela Roscetto,[a] Tiziana Angrisano,[a] Valerio Costa,[b] Mariassunta Casalino,[c] Konrad U. Förstner,[d] Cynthia M. Sharma,[d] Pier Paolo Di Nocera,[a] and Eliana De Gregorio[a]

Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università Federico II, Naples, Italy[a]; Institute of Genetics and Biophysics "Adriano Buzzati-Traverso," CNR, Naples, Italy[b]; Dipartimento di Biologia, Università Roma TRE, Rome, Italy[c]; and Research Center for Infectious Diseases, University of Würzburg, Würzburg, Germany[d]

Hfq is an RNA-binding protein known to regulate a variety of cellular processes by interacting with small RNAs (sRNAs) and mRNAs in prokaryotes. *Stenotrophomonas maltophilia* is an important opportunistic pathogen affecting primarily hospitalized and immunocompromised hosts. We constructed an *hfq* deletion mutant (Δ*hfq*) of *S. maltophilia* and compared the behaviors of wild-type and Δ*hfq S. maltophilia* cells in a variety of assays. This revealed that *S. maltophilia* Hfq plays a role in biofilm formation and cell motility, as well as susceptibility to antimicrobial agents. Moreover, Hfq is crucial for adhesion to bronchial epithelial cells and is required for the replication of *S. maltophilia* in macrophages. Differential RNA sequencing analysis (dRNA-seq) of RNA isolated from *S. maltophilia* wild-type and Δ*hfq* strains showed that Hfq regulates the expression of genes encoding flagellar and fimbrial components, transmembrane proteins, and enzymes involved in different metabolic pathways. Moreover, we analyzed the expression of several sRNAs identified by dRNA-seq in wild-type and Δ*hfq S. maltophilia* cells grown in different conditions on Northern blots. The accumulation of two sRNAs was strongly reduced in the absence of Hfq. Furthermore, based on our dRNA-seq analysis we provide a genome-wide map of transcriptional start sites in *S. maltophilia*.

Stenotrophomonas maltophilia is a Gram-negative environmental gammaproteobacterium that has been recognized as an important opportunistic pathogen in the last decade, affecting primarily hospitalized and immunocompromised hosts (3). *S. maltophilia* is frequently isolated from the lungs of cystic fibrosis patients, and its multidrug-resistant phenotype complicates the eradication of *S. maltophilia* infections (35). Factors that may contribute to the pathogenicity of *S. maltophilia* include a wide range of extracellular enzymes potentially involved in the colonization process, such as fibrolysin, lipases, and proteases (6), the ability to adhere to and form biofilms on epithelial cells (31), and replication and persistence in the lung (34). Deterioration of lung function associated with *S. maltophilia* infections has been demonstrated by infection assays carried out in mice (10).

Whole genome sequences of three *S. maltophilia* strains are available: (i) R551-3, a strain which was isolated from *Populus trichocarpa* (46); (ii) SKA14, which was isolated from the Baltic Sea (15); and (iii) K279a, a strain isolated from a cancer patient (6). Comparative genome-wide analyses revealed that the R551-3 and K279a genomes share a conserved backbone but contain different sets of genomic islands (GEIs) (33).

Hfq (host factor I protein) is a highly conserved RNA chaperone that was first identified as a protein required for the replication of the RNA phage Qβ in *Escherichia coli* (13). Hfq plays an important role in the fitness and virulence of many pathogenic bacteria, and *hfq* mutants often exhibit pleiotropic phenotypes, including defects in quorum sensing, growth rate, stress tolerance, and virulence (4). Hfq mainly functions as a posttranscriptional regulator by stabilizing small RNAs (sRNAs) and facilitating their interactions with mRNA targets (49).

Thus far, nothing is known about the role of Hfq and sRNAs in *S. maltophilia*. We constructed an in-frame deletion mutant of the *S. maltophilia hfq* gene and carried out several analyses to investigate the role of Hfq in this organism. Moreover, using RNA-sequencing-based transcriptome analyses, we also identified several *S. maltophilia* genes that are potentially regulated by Hfq and found that the accumulation of two intergenic sRNAs was impaired in the absence of Hfq. Furthermore, based on our differential RNA sequencing analysis (dRNA-seq) analysis, we annotate 1,030 putative transcriptional start sites in *S. maltophilia*.

MATERIALS AND METHODS

Bacterial strains. *S. maltophilia* strain K279a was kindly provided by M. B. Avison (University of Bristol, Bristol, United Kingdom). To construct the *hfq* deletion strain, DNA fragments located immediately upstream and downstream from the *S. maltophilia hfq* gene were amplified by PCR. Reaction products were digested with XbaI-BglII and BglII-XhoI, respectively, and the double insert was cloned into XbaI-XhoI-restricted pGEM vector DNA, yielding pGEM-Δ*hfq*. The 218-bp *hfq* gene deletion was confirmed by nucleotide sequencing. After digestion of pGEM-Δ*hfq* with XbaI and XhoI, the Δ*hfq* gene-containing fragment was cloned into the suicide vector pDM4, which contains the *sacBR* genes conferring sucrose sensitivity. Plasmid pDM4-Δ*hfq* was introduced into *Escherichia coli* S17-1 by transformation and subsequently mobilized to the *S. maltophilia* K279a strain via conjugation as described previously (22). pDM4-Δ*hfq* was integrated into the genome by homologous recombination, and the resulting merodiploid strain was selected on Luria-Bertani (LB) agar supplemented with chloramphenicol (100 μg ml^{-1}). Excision of the inte-

Received 30 April 2012 **Accepted** 18 August 2012
Published ahead of print 24 August 2012
Address correspondence to Eliana De Gregorio, edegrego@unina.it.
E.R. and T.A. contributed equally to this article.
Supplemental material for this article may be found at http://jb.asm.org/.

doi:10.1128/JB.00746-12

grated suicide vector could promote allelic exchange, which may either generate the deletion strain desired or restore the wild-type condition. Strains were first selected on LB agar supplemented with 10% sucrose to eliminate cells carrying integrated *sacBR* genes. Sucrose-resistant colonies were screened for chloramphenicol sensitivity to monitor excision of the suicide vector. The inactivation of the *hfq* gene in chloramphenicol-sensitive colonies was confirmed by PCR amplification.

Stress tolerance assays. Cultures were grown for 3 h at 37°C in LB broth, harvested by centrifugation, washed once with phosphate-buffered saline (PBS), and resuspended in water to an optical density at 600 nm (OD_{600}) of 1.0. Serial dilutions were spotted onto LB plates, supplemented with 5% ethanol, 5× NaCl (50 mg ml^{-1}), or 150 mM methyl viologen. Plates were incubated at 37°C for 48 h. The results shown are representative of at least three independent experiments.

Motility assays. Swimming, swarming, and twitching assays were performed using 0.3, 0.5, and 1% Mueller-Hinton (MH) agar plates, respectively. Swim plates were inoculated with toothpicks carrying bacteria grown overnight in LB agar plates at 37°C. Bacteria isolated from swim plates were inoculated onto swarm plates. Swim and swarm plates were incubated at 30°C for 24 h. To analyze twitching motility, bacteria were inoculated at the bottom of twitch plates. After incubation at 37°C for 24 h, the zone of motility at the agar-petri dish interface was measured by staining with Coomassie brilliant blue. The results shown are representative of at least three independent experiments.

Biofilm assays. Biofilm assays were performed as described previously (31). Overnight Trypticase soy broth (TSB) cultures were transferred to the wells of polystyrene microtiter plates. The plates were incubated at 37°C for 24 h and then stained with crystal violet. Solubilized crystal violet was determined spectrophotometrically at OD_{595}. The results shown are representative of at least three independent experiments.

***In vitro* antimicrobial sensitivity assay.** MICs were determined in MH medium by Etest (AB Biodisk), according to the manufacturer's instructions.

Adhesion on and internalization in IB3-1 cells. IB3-1 cells were grown at 37°C in LHC-8 medium supplemented with 5% fetal bovine serum in a 5% CO_2 atmosphere. Bacteria used to infect IB3-1 cells were grown in TSB at 37°C, washed in PBS, and resuspended in LHC-8 medium to an OD_{600} of 0.500, corresponding to about 5×10^8 CFU ml^{-1}. The concentration (CFU ml^{-1}) of each bacterial suspension used to infect cultured cells was determined.

IB3-1 cells were cultured in six-well polystyrene plates seeded with 5×10^5 cells/well and grown to confluence in LHC-8 medium. Before seeding, the wells were coated with 0.01 mg of human fibronectin ml^{-1}, 0.03 mg of bovine type 1 collagen ml^{-1}, and 0.01 mg of bovine serum albumin ml^{-1}. Monolayers were infected with approximately 5×10^7 K279a or Δ*hfq* cells, suspended in LHC-8 medium to obtain a multiplicity of infection (MOI) of 100, and kept at 37°C for 2 h. For adhesion assays, infected monolayers were washed with PBS to remove nonadherent bacteria and treated with 0.25% trypsin-EDTA. Cell lysates were recovered, serially diluted, and plated on MH agar. For internalization assays, infected monolayers were washed with PBS and further incubated for 2 h in LHC-8 medium supplemented with gentamicin (1 mg ml^{-1}) in order to kill extracellular bacteria. The cells were washed in PBS and lysed with 0.1% Triton X-100. Aliquots of cell lysates were serially diluted and plated to quantify viable intracellular bacteria.

Phagocytosis, intracellular survival, and replication in macrophages. RAW 264.7 macrophages were grown at 37°C in RAWGM1 (Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 20 mM HEPES) in a 5% CO_2 atmosphere. Macrophages, seeded in six-well plates 1 day prior to infection at 10^6 cells/well, were infected with an MOI of 100 and then incubated for 30 min at 37°C. Macrophages were washed with PBS, and phagocytosed bacteria were counted after 1 h of incubation in RAWGM1 containing gentamicin (1 mg ml^{-1}) to kill extracellular bacteria. To measure intracellular survival and replication, cells were washed with PBS and incubated in fresh medium containing 20 μg of gentamicin ml^{-1} for 2 and 24 h. Macrophages were washed and lysed, and viable bacteria were counted. Experiments were carried out in triplicates, and the data are representative of two independent experiments.

Virulence assay in *Galleria mellonella* larvae. Insect infections with *S. maltophilia* were carried out essentially as previously described (39). Briefly, bacterial cultures were centrifuged, resuspended in 10 mM $MgSO_4$ supplemented with 0.15 mg of ampicillin/ml, and serial 10-fold dilutions were prepared. Ten larvae were injected with 5 μl of each dilution (containing from ~10^7 to 0 CFU). Larvae were incubated at 37°C, and the number of dead or alive larvae was scored every 24 h over a 72 h period. Larvae were considered dead when they displayed increased melanization and were not mobile.

RNA isolation. RNA was isolated from *S. maltophilia* wild-type and Δ*hfq* cultures at different growth phases. Culture aliquots were removed and mixed with a 0.2 volume of stop mix (5% water-saturated phenol, 95% ethanol), snap-frozen in liquid nitrogen, and stored at −80°C until RNA isolation. After thawing on ice, bacteria were centrifuged, and RNA was isolated using the TRIzol method, followed by treatment with DNase I (43).

Reverse transcription assays. Reverse transcriptase PCR (RT-PCR) analyses were carried out by reverse transcription of *S. maltophilia* total RNA using random primers and avian myeloblastosis virus (AMV) reverse transcriptase (Roche). The resulting cDNA was amplified using pairs of gene-specific oligonucleotides. For control reactions, RNA without RT or chromosomal DNA was used as a template. Real-time quantitative PCR (qRT-PCR) was performed with gene-specific primers using a Chromo4 real-time thermocycler (Bio-Rad). Then, 2 μl of cDNA (10 ng/reaction) was used as a template for qPCRs with Power SYBR green PCR master mix (Applied Biosystems) and primers at a 500 nM final concentration. The real-time cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. No-template and no-RT controls were included for each primer set and template. Melting-curve analysis verified that each reaction contained a single PCR product. To compare transcript levels, the amounts of transcript were normalized to 16S rRNA amounts. Primer extension analyses were performed as previously described (7).

Northern blot analyses. For Northern blot analyses, RNA was isolated from *S. maltophilia* wild-type and Δ*hfq* cultures under four different conditions: (i) condition 1, log phase, LB broth, 37°C; (ii) condition 2, stationary phase, LB broth, 37°C; (iii) condition 3, log phase, LB broth, 28°C; and (iv) condition 4, log phase, synthetic cystic fibrosis medium (SCFM), 37°C. A 10-μg portion of total RNA was separated on 8% polyacrylamide–8 M urea gels and transferred to Hybond-N^+ membranes by electroblotting. Membranes were hybridized in hybridization buffer (4× SSC, 1× Denhardt's solution, 0.5× sodium dodecyl sulfate) at 42°C with gene-specific ^{32}P-end-labeled DNA oligonucleotides. Membranes were subsequently washed and rehybridized to a ^{32}P-end-labeled oligonucleotide complementary to *S. maltophilia* 5S rRNA to normalize data.

Construction of cDNA libraries for dRNA-seq and sequencing. Sample processing, library construction, and deep sequencing analysis were performed as previously described (40). Briefly, total RNA was extracted from K279a and Δ*hfq* cultures in exponential growth, and primary transcripts were enriched by a selective degradation of RNAs containing a 5′ monophosphate (5′P) by treatment with 5′P-dependent terminator exonuclease (Epicentre). Specifically, equal amounts of RNA were incubated for 60 min at 30°C with terminator exonuclease (TEX-treated library) or in buffer alone (untreated library). After phenol-chloroform extraction, the RNA was precipitated, and treated with 1 unit TAP (tobacco acid pyrophosphatase; Epicentre) for 1 h at 37°C to generate 5′ monophosphates for linker ligation and again purified by organic extraction and precipitation as described above. cDNA libraries for Illumina GAIIx sequencing were constructed by Vertis Biotechnology AG, Germany. First, TEX−/+-treated RNA samples were poly(A)-tailed using

poly(A) polymerase, followed by ligation of an RNA adapter to the 5′ P of the RNA. First-strand cDNA synthesis was performed using an oligo(dT)-adapter primer and Moloney murine leukemia virus RNase H reverse transcriptase. The resulting cDNAs were PCR amplified to about 20 to 30 ng/μl using a high-fidelity DNA polymerase. Incubation temperatures were 42°C for 20 min, ramp to 55°C, followed by 55°C for 5 min. Each library contains a specific barcode sequence, which is attached to the 5′ ends of the cDNAs. The primers used for PCR amplification were designed for amplicon sequencing according to the instructions of Illumina/Solexa. The following adapter sequences flank the cDNA inserts (NNNN indicates the barcode sequence): 5′-end (53 bases), 5′-AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGACGATC-NNNN-3′; and 3′-end (45 bases), 5′-CAAGCAGAAGACGGCATACGATTTTTTTTTTTTTTTTTTTTTTTTT-3′. The combined length of the flanking sequences is 98 bases.

The resulting cDNA libraries were sequenced on an Illumina GAII machine. For K279a and Δ*hfq* untreated libraries, 1,718,635 and 1,499,053 reads were obtained, respectively. For the K279a and Δ*hfq* TEX-treated libraries, 772,866 and 100,6704 reads were obtained, respectively. The sequencing data have been deposited in the NCBI Gene expression omnibus (GEO) database with the accession no. GSE39705.

Bioinformatic analysis. The sequences of the obtained reads were quality trimmed using the program fastq_quality_trimmer (part of the FASTX toolkit) with a cutoff score of 20. Poly(A) tail sequences were removed from the 3′ end of the sequences, and the resulting sequences were filtered by length. Sequences shorter than 12 nucleotides (nt) were discarded in this step. The filtered reads were mapped to the *S. maltophilia* K279a genome (accession no. AM743169.1) via Segemehl (16). Based on these read mappings, coverage plots indicating the number of mapped reads per nucleotide were generated and then visualized in the Integrated Genome Browser (28). Comparative expression level analysis was performed using the R library DESeq (2).

Annotation of TSS. Transcriptional start sites (TSS) were manually annotated by inspection of sequenced regions which mapped upstream of annotated open reading frames (ORFs). All 5′ ends that showed higher cDNA coverage in the TEX+ library than in the TEX− library in at least of the two strains were considered putative TSS (40). Only sequencing reads initiating with the same nucleotide in at least three libraries have been taken into account. Based on custom-made Python scripts, TSS were automatically associated with genes and allocated to different classes. TSS located ≤300 nt upstream of a gene on the same strand were classified as "primary" TSS if they had the shortest distance to the gene. Any other TSS in that range and orientation was classified as a "secondary" TSS. A TSS could have only one such 5′-end association with a gene. TSS positioned inside a gene in sense direction were called "internal." TSS on the opposite strand and located in a range of ≤100 nt upstream and 100 nt downstream of a gene were classified as "antisense." A TSS without any gene association was called an "orphan."

RESULTS

Genomic location of the *S. maltophilia hfq* gene. The Smlt1736 gene of *S. maltophilia* strain K279a encodes a 91-amino-acid protein that is annotated as Hfq and conserved in strains R551-3 and SKA14. Moreover, Smlt1736 is 89% identical to the *E. coli* and *Salmonella enterica* serovar Typhimurium Hfq proteins and exhibits extensive similarity at the N terminus to Hfq encoded by other bacterial species (Fig. 1A). The amino acid residues Gln8, Phe39, Lys56, and His57, which have been shown to be involved in RNA binding (37), are conserved in the *S. maltophilia* Hfq homolog (Fig. 1A). As in *E. coli*, the *S. maltophilia hfq* gene is located between the *miaA* and *hflX* genes, encoding the tRNA δ-(2)-isopentenylpyrophosphate transferase and the GTP-binding protein HflX, respectively (Fig. 1B). This gene organization is conserved in many bacterial species. In *E. coli*, the three genes are part of a large operon exhibiting a complex transcriptional organization (48). To assess whether *hfq* is transcribed independently or along with flanking sequences, RT-PCR experiments with primers spanning the adjacent *miaA* and *hflX* genes were performed. The results showed cotranscription of *hfq* and *hflX* only (Fig. 1C). To identify the transcriptional start site of the *hfq* transcript, primer extension analyses were carried out (Fig. 1D). Two bands of 218 and 310 nt were the predominant signals detected both in exponentially growing and stationary phase cells. The 310-nt band corresponds to transcripts initiating within the *miaA* gene, as shown in *E. coli* (48), and the 218-nt band presumably corresponds to processed transcripts (see RNA sequence data below).

Construction of the *S. maltophilia* Δ*hfq* mutant. To investigate the role of Hfq in *S. maltophilia*, we constructed a K279a strain derivative carrying an in-frame deletion of the *hfq* gene region encoding amino acids 8 to 83. The Δ*hfq* isogenic mutant was obtained by insertion of a suicide vector, which was introduced into K279a via conjugation, followed by allele replacement mutagenesis (22; see also Materials and Methods). The deletion was confirmed by PCR sequencing, and the loss of *hfq* gene expression was verified by RT-PCR experiments (Fig. 1C). RT-PCR analyses also showed that the *hfq* deletion had no polar effect on the transcription of the downstream *hflX* gene (data not shown).

Growth characteristics, stress tolerance, and antibiotic susceptibility of the Δ*hfq* mutant. An impaired growth of *hfq* mutants in certain media has been reported for several species (12, 23, 38, 42, 48). The growth rates of the Δ*hfq* mutant and K279a wild-type strains at 37°C were only slightly different in Luria broth and in synthetic cystic fibrosis medium (SCFM) (29), with the Δ*hfq* mutant reaching a lower saturation density than strain K279a. In contrast, the growth of the mutant was severely reduced at 28°C (see Fig. S1 in the supplemental material), and Δ*hfq* cells had a doubling time (55 min) significantly longer than that of K279a cells (42 min).

Hfq has been shown to be involved in stress tolerance in many pathogens (4). The role of Hfq in *S. maltophilia* stress response was tested under various conditions. *S. maltophilia* was exposed to stress by adding ethanol, NaCl (osmotic stress) or methyl viologen (oxidative stress) to the medium. However, none of these stress conditions affected the growth of wild-type and Δ*hfq* cells (see Fig. S2 in the supplemental material).

S. maltophilia exhibits high-level intrinsic resistance to a variety of structurally unrelated antibiotics (3). The susceptibilities of K279a and Δ*hfq* cells to 23 different antibiotics are reported in Table 1. The mutant was less susceptible to tetracycline, chloramphenicol, and ciprofloxacin but more susceptible to tobramycin and amikacin than strain K279a. A similar phenotype was exhibited by the *S. maltophilia* D457R strain, in which the SmeDEF efflux pump genes are overexpressed (1, 36). Moreover, the Δ*hfq* mutant showed an increased sensitivity to colistin. This is in agreement with results indicating that the resistance of uropathogenic *E. coli* strains to polymyxin B is mediated by Hfq (18).

Deletion of *hfq* leads to impaired motility and biofilm formation. Motility and biofilm formation have been shown to require Hfq in several species (18, 42, 44, 45). We examined the effect of Hfq on the swarming, swimming, and twitching ability of *S. maltophilia*. Swimming and swarming of *S. maltophilia* cells were monitored on 0.3 and 0.5% agar plates, respectively. In both cases,

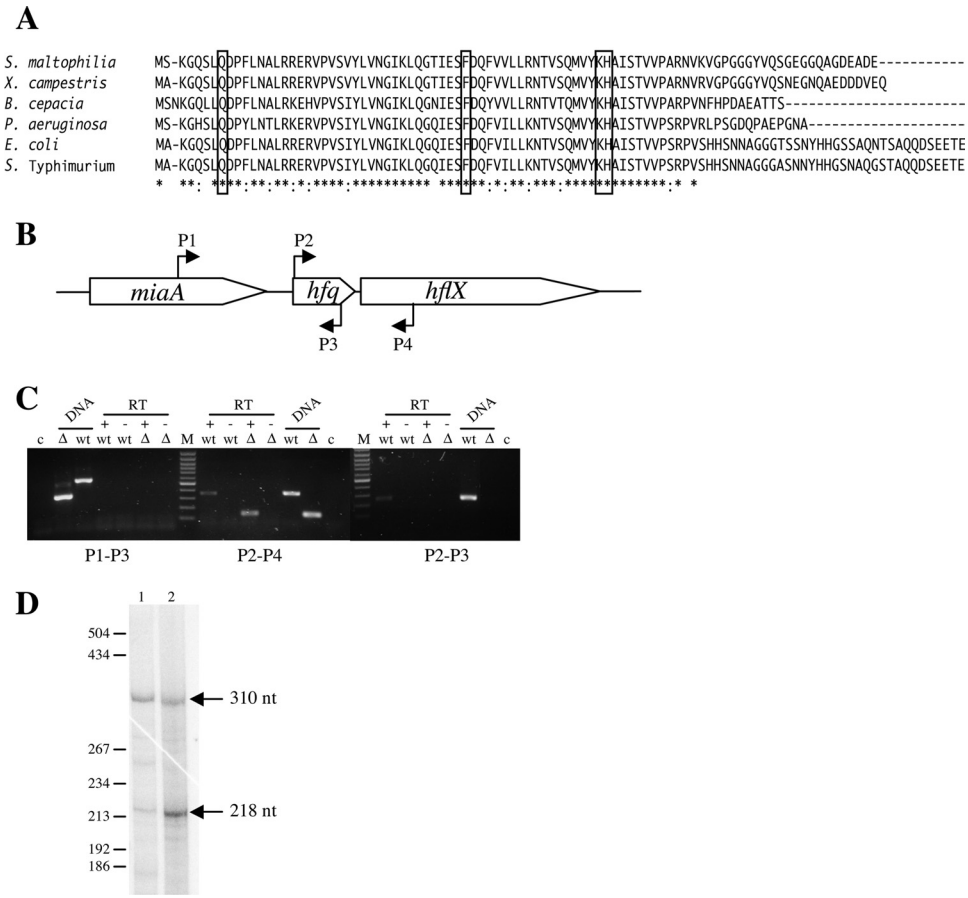

FIG 1 Hfq proteins and the *hfq* locus. (A) Alignment of bacterial Hfq proteins. Residues identical in all strains are indicated by asterisks, conserved substitutions are indicated by double dots. Amino acids implicated in binding of RNA are boxed. (B) Organization of the *S. maltophilia hfq* locus. The relative positions of oligonucleotides P1 to P4 used as primers for RT-PCR are indicated by the arrows. (C) RT-PCR analysis of *hfq* transcripts. The primer pair used in each experiment is indicated at the bottom of the gel. PCR amplification was performed without DNA template (c), with genomic DNA isolated from K279a or the Δ*hfq* mutant (DNA), with RNA from isolated K279a or the Δ*hfq* mutant after reverse transcription (RT+), or with RNA isolated from K279a and the Δ*hfq* mutant without reverse transcription (RT−). wt, K279a; Δ, Δ*hfq* mutant; M, 100-bp plus DNA ladder. (D) Primer extension analysis. The ^{32}P-5-end-labeled primer complementary to the coding regions of the *hfq* gene was hybridized to total RNA purified from the K279a strain grown at 37°C in LB medium to exponential phase (lane 1) or stationary phase (lane 2). Annealed primer moieties were extended, in the presence of nucleoside triphosphates, by AMV RT. Reaction products were electrophoresed on a 6% polyacrylamide–8 M urea gel. Major reaction products are marked by arrows. The numbers to the left of the autoradiogram refer to the size in nucleotides of coelectrophoresed DNA molecular size markers.

the mutant strain was less motile than K279a (Fig. 2A). In contrast, the twitching motilities of the mutant and K279a strains were roughly the same (Fig. 2A). The involvement of Hfq in biofilm development was tested by growing K279a and Δ*hfq* cells at 37°C in TSB medium in polystyrene microtiter plates. In these assays, the Δ*hfq* mutant showed a 2-fold reduction in biofilm formation (Fig. 2B, black bars). Also in SCFM, less biofilm formation was observed for the Δ*hfq* strain (Fig. 2B, gray bars).

Adhesiveness, internalization, and survival in epithelial cells and macrophages. *S. maltophilia* is able to adhere to and penetrate epithelial respiratory cells (9, 31). Wild-type and Δ*hfq* cells were used to infect IB3-1 bronchial epithelial cells at an MOI of 100, and the number of CFU was determined after 2 h. Deletion of *hfq* led to a 30-fold reduction in bacterial adhesion (Fig. 3A, gray bars). After gentamicin treatment for 1 h, the number of intracellular bacteria was determined 2 h postinfection. The Δ*hfq* strain was 10-fold less invasive than the K279a wild type (Fig. 3A, black bars). The assays were repeated using RAW 264.7 murine macrophages as the recipient (23, 38, 42). No significant difference in the abilities of wild-type and Δ*hfq* cells to be internalized and survive inside macrophages was observed (Fig. 3B, black and light gray bars). In contrast, the intracellular replication of the Δ*hfq* mutant was 5-fold less efficient (Fig. 3B).

Wax worm *G. mellonella* larvae were infected with K279a and Δ*hfq* cultures as previously described (39). According to the 50% lethal dose values, the pathogenicities of the two strains were similar (data not shown).

Differentially expressed genes in the *S. maltophilia hfq* mutant. To identify genes that are regulated by Hfq in *S. maltophilia*, the transcriptomes of K279a and the *hfq* mutant were analyzed by differential RNA-sequencing (dRNA-seq), which is selective for the 5′ ends of primary and processed transcripts. Total RNA was extracted from K279a wild-type and Δ*hfq* cultures in exponential growth, split in two halves, one of which was treated with 5′-phosphate dependent terminator exonuclease (TEX). Afterward, both halves were converted into cDNA libraries as described previously (40). Overall, for each strain, two cDNA libraries, one covering both primary (5′PPP RNAs) and processed (5′P RNAs)

TABLE 1 Susceptibilities of *S. maltophilia* K279a and Δ*hfq* mutant cells to different antimicrobial agents

Antimicrobial agent	MIC (μg/ml)	
	K279a	Δ*hfq* mutant
Ceftazidime	>256	>256
Aztreonam	>256	>256
Piperacillin	>256	>256
Piperacillin-tazobactam	>128	>128
Meropenem	>32	>32
Gentamicin	16	16
Levofloxacin	2	1.5
Televancin	>32	>32
Linezolid	>256	>256
Daptomycin	>256	>256
Rifampin	24	24
Imipenem	96	192
Ciprofloxacin	4	16
Netilmicin	24	48
Chloramphenicol	6	24
Fosfomycin	64	192
Trimethoprim-sulfamethoxazole	0.75	6
Tetracycline	2	16
Tigecycline	0.19	1
Vancomycin	64	96
Amikacin	16	6
Tobramycin	24	12
Colistin	6	2

transcripts (untreated library) and the other enriched in primary transcripts (treated library), were sequenced.

For K279a and Δ*hfq* untreated libraries, 1,718,635 and 1,499,053 reads, respectively, were obtained. For K279a and Δ*hfq* TEX-treated libraries, 772,866 and 1,006,704 reads, respectively, were obtained. The number of reads, the percentage of mapped reads, and the amount of single reads of both treated and untreated libraries are reported in Table S1 in the supplemental material.

Treatment of the RNA with TEX prior to sequencing enables identification of TSS. As previously described for *Helicobacter pylori* (40), TSS were manually annotated by inspection for characteristic enrichment patterns in the TEX+ libraries toward the 5′ ends of transcripts. All 5′ ends that showed higher cDNA coverage in the TEX+ than in the TEX− library in at least of the two strains were considered putative TSS. Only sequencing reads initiating with the same nucleotide in at least three libraries have been taken into account. In total, 1,030 putative TSS were annotated (listed in Table S2 in the supplemental material).

Incidentally, dRNA-seq analyses confirmed the *hfq* TSS within the *mia* gene identified by primer extension in Fig. 1. The lack of a TSS corresponding to the shorter 218-nt *hfq* RNA, also detected in the Fig. 1 experiment, suggests that this band is a processed RNA species.

Analysis of the RNA-seq data with the DESeq package (2) revealed 63 candidate genes with at least a 2-fold difference in relative transcript levels (with a *P* value of <0.05; see Table S3 in the supplemental material). As summarized in Table 2, deletion of *hfq* leads to decreased expression of 35 genes, and increased expression of 28 genes. Downregulated genes are involved in the formation of fimbriae, or control the synthesis (*fliA* gene) or the assembly (*fliM*, *fliI*, and *fliE* genes) of flagellar proteins. Others encode proteins with diverse functions, such as a disulfide isomerase, an aquaporin Z, and a methyl-accepting chemotaxis receptor. The set of genes upregulated in Δ*hfq* cells is also heterogeneous. Many encode surface proteins (transmembrane and outer membrane proteins and type IV secretion system components).

Changes in the accumulation of selected RNAs detected by dRNA-seq were experimentally validated by qRT-PCR analyses. We selected 10 genes, five downregulated (Smlt0706, Smlt0708, Smlt2127, Smlt2314, and Smlt3559) and five upregulated (Smlt1742, Smlt3003, Smlt3461, Smlt4085, and Smlt4179) in the *hfq* mutant. The results of PCR analyses all confirmed our dRNA-seq data (see Table S3 in the supplemental material).

Identification of *S. maltophilia* small RNAs. Hfq is an RNA-binding protein shown to interact with, and regulate the function of several sRNAs (49). The manual inspection of the RNA sequencing data led to the identification of 60 sRNA candidates, denoted SmsR (*S. maltophilia* sRNA). Of these, 12 partly overlap coding regions (Table 3). Candidates include conserved housekeeping regulatory RNAs, such as RNase P RNA, SRP RNA, tmRNA, and 6S RNA, and the 16-nt 6S RNA-associated product RNAs (pRNAs), which are transcribed from 6S RNA as a tem-

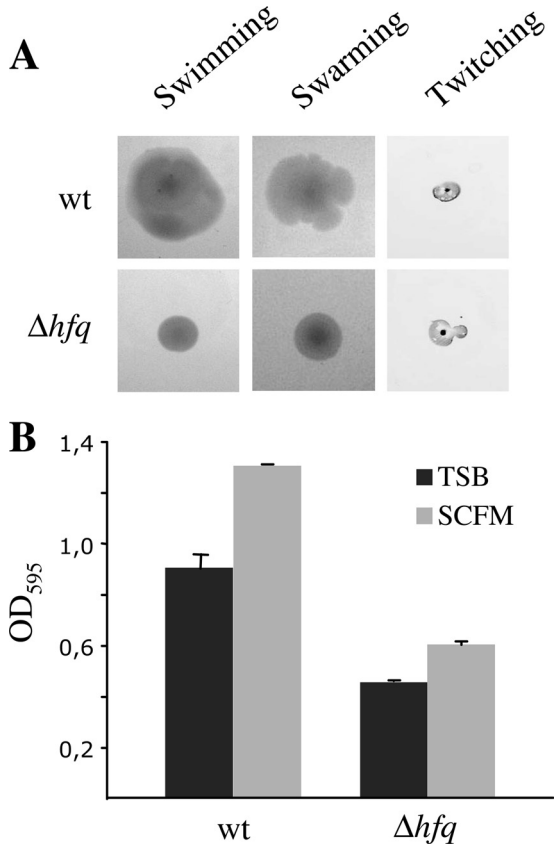

FIG 2 Mutation of the *hfq* gene affects the motility and biofilm formation of *S. maltophilia*. (A) K279a (wt) and Δ*hfq* strains were cultured on different types of agar plates at 30°C, and motility was monitored 72 h later. The images are representative of several experiments. (B) Biofilm formation in polystyrene microtiter plate by K279a (wt) and Δ*hfq* strains was measured using crystal violet staining after 48 h of growth in TSB or SCFM medium at 37°C. Experiments were performed at least three times, and the standard deviation is shown. OD_{595}, optical density at 595 nm.

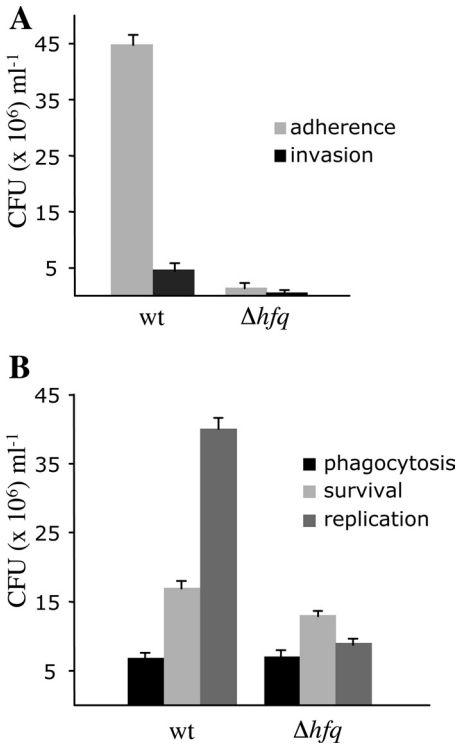

FIG 3 Infection of epithelial cells and macrophages. (A) Adhesion to IB3-1 cells and internalization of K279a (wt) and Δ*hfq* cells. IB3-1 monolayers were infected with ~100 bacteria per cell for 2 h. CFU were determined for adherent (gray bars) or internalized (black bars; internalization was measured after 1 h of treatment with gentamicin) bacteria by culturing IB3-1 cell lysates on TSA plates for 48 h. (B) Infection of RAW 264.7 macrophages. Cells were infected with ~100 bacteria per cell for 30 min. After treatment with gentamicin for 1 h, phagocytosis, intracellular survival, and replication in macrophages were determined at 0, 2, and 24 h postinfection, respectively. The data in panels A and B are means of three independent experiments, each performed in triplicate. Error bars indicate the standard deviations.

plate and destabilize RNA polymerase-6S RNA interactions (50). Three candidates in Table 3 are predicted to encode potential *cis*-regulatory RNA riboswitches regulated by adenosyl cobalamin (AdoCbl; SmsR53), glycine (Gly; SmsR54), and thiamine pyrophosphate (TPP; SmsR55). Three candidate RNAs (SmsR2, SmsR17, and SmsR19) are encoded by repeated sequences scattered throughout the K279a genome. Multiple copies of some sRNA genes have also been identified in other bacteria (5, 20).

Twenty candidate sRNAs were selected for Northern blot analyses based on high read numbers according to our dRNA-seq data. Hybridization signals were detected for only 16 candidates (Table 3). Two of them, SmsR50 and SmsR51, are present in five and six copies in K279a, respectively, mapping in the antisense region of the 5′ untranslated region of the transposase genes of the ISStma6 and ISStma7 insertion elements. Six sRNAs (SmsR8, SmsR26, SmsR38, SmsR43, SmsR50, and SmsR51) were constitutively expressed in all conditions tested (Fig. 4). SmsR51 appears to accumulate at levels 2-fold higher in the Δ*hfq* mutant. In contrast, the levels of all other sRNAs are unaltered by the *hfq* deletion (Fig. 4).

One of the selected sRNAs is the housekeeping 6S RNA. The accumulation of 6S RNAs is growth dependent, since hybridization signals were increased 2-fold in stationary phase in both wild-type and Δ*hfq* cells. As shown in *E. coli* (25), the deletion of *hfq* had no major effect on the accumulation of 6S RNA (Fig, 5). Expression of three sRNAs (SmsR13, SmsR35, and SmsR39) varied when cells were grown in SCFM rather than in LB broth. The levels of SmsR13 were dramatically (27-fold increase) influenced by SCFM. However, the accumulation of this RNA is not affected by the *hfq* deletion (Fig. 5A). The levels of SmsR35 increased 5-fold in the K279a cells grown in SCFM but decreased 3-fold when the mutant was grown in the same medium. The levels of SmsR39 increased only 2-fold in the SCFM, but no change was observed in the Δ*hfq* mutant (Fig. 5A).

The accumulation of another six sRNAs was affected in the Δ*hfq* mutant (Fig. 5B). The levels of four of them (SmsR3, SmsR6, SmsR15, and SmsR41) were reduced ~2-fold in the mutant. In contrast, the levels of SmsR20 and SmsR36 were significantly reduced. Hybridization signals for SmsR20 and SmsR36 in Fig. 5 are faint. However, prolonged exposure of the autoradiogram and subsequent quantification with the QuantityOne software revealed that SmsR20 and SmsR36 levels were reduced 10- and 5-fold, respectively, in the Δ*hfq* mutant (Fig. 5B). These data were fully supported by dRNA-seq analyses.

To check for conservation of our sRNA candidates, sequences homologous to the candidate sRNAs were searched in the genomes of different *S. maltophilia* strains or other species by BLAST (Table 3). Several sRNAs seem to be species specific, since homologues were found only in *S. maltophilia*. Ten are specific for strain K279a, since they map in genomic islands present in this strain but are missing in the SKA14, R551-3, and JV3 strains. Sequences homologous to SmsR12 and SmsR15 were detected in many bacteria. Sequences homologous to half (34/60) of the remaining sRNAs were found only in *Xanthomonas* species (Table 3).

DISCUSSION

Hfq is a protein of pleiotropic function and has been shown to be a key component of diverse cellular processes and regulatory circuits (4). In the present study we analyzed the functional properties of Hfq in the emerging pathogen *S. maltophilia* in a variety of assays. As observed in many species, growth was poorly impaired by inactivation of the *hfq* gene. In contrast, the intrinsic multidrug resistance of *S. maltophilia* was severely affected upon deletion of *hfq* (Table 1). In *E. coli*, an Hfq-dependent sRNA called MgrR has been shown to regulate antibiotic resistance (27). Perhaps also in *S. maltophilia* some of the newly identified sRNAs have a role in regulation of multidrug resistance. Biofilm formation was significantly impaired in the Δ*hfq* mutant. Hfq is implicated in biofilm development in many bacteria, such as uropathogenic *E. coli* (18), *Pseudomonas fluorescens* (51), and *S. enterica* serovar Typhimurium (26). Recent studies reported that the Hfq-dependent sRNAs McaS, RprA, GcvB, OmrA/B, ArcZ, and SdsR are implicated in the regulation of the major biofilm regulator CsgD (17, 24, 26, 47). Swimming and swarming motility were also reduced, but twitching was not affected (Fig. 2). The same phenotype was displayed by a *Neisseria gonorrhoeae* Δ*hfq* mutant (11). Hfq has a role in adherence to and invasion of epithelial cells and seems to be crucial for the ability of *S. maltophilia* to replicate within macrophages (Fig. 3). Results for wild-type and Δ*hfq* cells were indistinguishable in the *G. mellonella* larva virulence assay. The lack of an observable effect in this system is not surprising, since the pathogenicity of *S.*

TABLE 2 Genes differently expressed in *S. maltophilia* K279a and Δ*hfq* cells

Locus	Gene	Gene product	KEGG pathway[a]
Genes significantly downregulated in the *hfq* mutant			
Smlt0140		Putative phosphoglycerate mutase family protein	
Smlt0206	*dhaA*	Putative haloalkane dehalogenase	Metabolism
Smlt0268	*mmsB*	Putative 3-hydroxyisobutyrate dehydrogenase	Metabolism
Smlt0681	*dsbC*	Putative thiol:disulfide interchange protein DsbC	Environmental information processing
Smlt0706	*smf1*	Putative fimbrial adhesin protein	Cellular processes
Smlt0707		Putative pili chaperone protein	
Smlt0708	*mrkC*	Putative outer membrane usher protein MrkC precursor	Cellular processes
Smlt0709		Putative fimbria adhesin protein	
Smlt0710	*wecB*	Putative UDP *N*-acetylglucosamine 2-epimerase	Metabolism
Smlt1134		Putative DNA transport competence protein	
Smlt1163		Hypothetical protein	
Smlt1306		Putative MFS family transmembrane transporter	
Smlt1408		Chromosome replication initiation inhibitor protein	
Smlt1736	*hfq*	Host factor I protein	Genetic information processing
Smlt2083		Putative transmembrane protein	
Smlt2127	*murB*	UDP *N*-acetylenolpyruvoyl-glucosamine reductase	Metabolism
Smlt2270	*fliA*	Putative RNA polymerase sigma factor for flagellar regulon	
Smlt2282	*fliM*	Flagellar motor switch protein FliM	
Smlt2310	*flgI*	Flagellar basal body P-ring protein	
Smlt2314	*flgE*	Flagellar hook protein FlgE	Cellular processes
Smlt2362		Hypothetical protein	
Smlt2558	*fruA*	Putative PTS system fructose-specific transporter subunit IIBC	Metabolism
Smlt2954	*mcpA*	Putative methyl-accepting chemotaxis protein	Environmental information processing
Smlt3161	*qbsG*	Putative kynurenine 3monooxygenase, siderophore biosynthesis	Metabolism
Smlt3301	*argG*	Argininosuccinate synthase	Metabolism
Smlt3314	*ubiG*	3-Demethylubiquinone 9 3-methyltransferase	Metabolism
Smlt3496		Putative glyoxalase/bleomycin resistance protein	Metabolism
Smlt3559	*aqpZ*	Putative aquaporin Z	Environmental information processing
Smlt3560		Hypothetical protein	
Smlt3691	*copA2*	Putative copper resistance protein	
Smlt3746		Putative ACR family protein	
Smlt3877		Putative transmembrane ion transporter	
Smlt3889		Hypothetical protein	
Smlt4268	*aceK*	Bifunctional isocitrate dehydrogenase kinase/phosphatase protein	Cellular processes and signaling
Smlt4409		Putative exported aminotransferase class V	
Smlt4528		Hypothetical protein	
Genes significantly upregulated in the *hfq* mutant			
Smlt0134	*hipA*	Putative survival protein HipA	Metabolism
Smlt0347		Metallobetalactamase family protein	
Smlt0396		Putative oxidoreductase	
Smlt0694	*pefL*	Putative general secretion pathway protein l	Environmental information processing
Smlt0717		Hypothetical protein	
Smlt0833		Hypothetical protein	
Smlt0953		Putative transmembrane protein	
Smlt1426		Outer membrane receptor FepA	Cellular processes and signaling
Smlt1445		Putative TetR family transcriptional regulator	
Smlt1476		Putative transmembrane protein	
Smlt1720		Putative Smr DNA repair family protein	
Smlt1742	*recX*	Recombination regulator RecX	
Smlt2477		Putative transmembrane protein	
Smlt3003		Putative type IV secretion system conjugal transfer protein Virb11	Environmental information processing
Smlt3033		Putative ankyrin repeat-containing protein	
Smlt3139		Hypothetical protein	
Smlt3461		Putative cell wall hydrolase	
Smlt3591		Putative transmembrane protein	
Smlt3660		Putative transmembrane protein	
Smlt3671	*pilJ*	Putative pilus biogenesis protein PilJ/methyl accepting chemotaxis protein	Environmental information processing
Smlt3696		Putative exported esterase	
Smlt3823		Putative transmembrane protein	
Smlt3911		Hypothetical protein	
Smlt4085		Putative transmembrane protein	
Smlt4179		Putative LysM family cell wall degradation protein	
Smlt4182		Putative fimbrial protein	
Smlt4389		Hypothetical protein	
Smlt4663		Putative outer membrane multidrug efflux protein	

[a] KEGG, Kyoto Encyclopedia of Genes and Genomes.

TABLE 3 Predicted sRNAs in *S. maltophilia* K279a

Name[a]	GEI[b]	Orientation[c]			Coordinates		Size (nt)		Conservation[d]				
		1	2	3	First	Last	RNA-seq	Northern	K	R	J	S	Others
sRNAs in intergenic region													
SmsR1	K1	<	>	<	30885	31076	191		+	–	–	–	–
SmsR2		>	>	>	95342	95408	66		+	+	+	+	–
SmsR3		>	>	>	98735	98831	96	90	+	+	+	+	–
SmsR4		<	<	<	200110	200262	152		+	+	+	+	–
SmsR5		>	<	<	272787	272878	91		+	–	–	–	–
SmsR6	K7	<	<	<	306561	306810	249	200, 100, 60	+	–	–	–	–
SmsR7		<	>	<	335444	335484	40		+	+	+	+	–
SmsR8		<	>	>	485142	485202	60	80, 57	+	+	+	+	A
SmsR9		<	<	<	611471	611540	69		+	+	+	+	A
SmsR10		>	>	<	875735	875806	71		+	+	+	+	A
SmsR11		<	<	>	1113554	1113664	110		+	+	–	–	–
SmsR12		>	<	<	1324322	1324417	95		+	–	–	–	H, L, P
SmsR13		>	>	>	1575836	1575994	158	147	+	+	+	+	A
SmsR14	K17	>	<	<	1722405	1722512	107		+	–	–	–	–
SmsR15		>	>	>	1724421	1724463	42	50	+	+	+	+	F, M, N, O
SmsR16		>	<	>	2002640	2002797	157		+	–	–	–	–
SmsR17		>	>	>	2046156	2046264	108		+	+	+	+	–
SmsR18		>	>	<	2075384	2075462	78		+	–	–	–	–
SmsR19		>	<	<	2216488	2216559	71		+	+	+	+	–
SmsR20		<	<	<	2344368	2344465	97	95	+	+	+	+	–
SmsR21		>	>	<	2401937	2402061	124		+	+	+	+	–
SmsR22	K27	<	>	>	2595533	2595634	101		+	–	+	–	A
SmsR23		<	>	>	2729432	2729599	167		+	+	+	+	–
SmsR24		<	<	<	2901942	2902011	69		+	+	+	+	–
SmsR25	K32	>	>	<	3092608	3092797	189		+	–	–	–	–
SmsR26		>	>	>	3139690	3139767	77	70	+	+	+	+	A
SmsR27		>	>	<	3367762	3368000	238		+	+	+	+	–
SmsR28		>	<	<	3701132	3701271	139		+	+	+	+	–
SmsR29		>	>	<	3713874	3713932	58		+	+	+	+	–
SmsR30		>	<	<	3714110	3714185	75		+	+	+	+	–
SmsR31		>	<	<	3743858	3743956	98		+	+	+	+	–
SmsR32		>	<	<	3913088	3913228	140		+	–	–	–	–
SmsR33		<	<	<	3923300	3923369	69		+	+	+	+	–
SmsR34		<	>	>	3957424	3957513	89		+	+	+	+	D
SmsR35		<	>	<	3975579	3975723	144	130, 90	+	+	+	+	–
SmsR36		<	>	<	3998834	3998935	101	90	+	+	+	+	–
SmsR37		<	>	>	4225415	4225495	80		+	+	+	+	–
SmsR38		<	<	<	4320478	4320576	98	100	+	+	+	+	A
SmsR39		<	<	<	4710815	4710928	113	110	+	+	+	+	A
sRNAs antisense to ORF													
SmsR40		>	<	>	246414	246509	95		+	+	+	+	–
SmsR41	K7	<	>	<	302539	302676	137	130	+	–	–	–	E
SmsR42	K11a	>	<		637514	637591	77		+	–	–	–	–
SmsR43	K17	>	<	>	1721111	1721239	128	100, 110	+	–	–	–	–
SmsR44		<	>		1758616	1758741	125		+	+	+	+	–
SmsR45		>	>	<	3215971	3216059	88		+	–	–	–	
SmsR46		>	<		4003714	4003826	112		+	+	+	+	–
SmsR47	K34	>	>	<	4277471	4277617	146		+	–	–	–	–
SmsR48		>	<	>	4726523	4726671	148		+	+	+	+	–
sRNAs sense to ORF													
SmsR49	K25	<	<		2514496	2514606	110		+	–	–	–	–
sRNAs antisense to the 5′ end of transposase genes													
SmsR50		–[e]					162	170	+	+	–	–	B, C
SmsR51		–[f]					158	160	+	+	–	–	B, C
SmsR52		–[g]					106		+	+	–	–	D
Riboswitches													
SmsR53		<	<	>	596373	596550	177		+	+	+	+	A, G
SmsR54		<	>	>	3630332	3630513	181		+	+	+	+	A, F
SmsR55		<	<	>	4001631	4001784	153		+	+	+	+	A, F
Known sRNAs													
RNase P		>	>	<	778453	778836	383		+	+	+	+	A, F, G, I
SRP RNA		>	<	>	1055518	1055726	208		+	+	+	+	A, F
tmRNA		>	<	>	1945039	1945390	351		+	+	+	+	A, F
6S RNA		>	>	>	3958091	3958322	231	234, 192	+	+	+	+	A, F
pRNA		>	<	>	3958125	3958140	15		+	+	+	+	A, F

[a] Underlined sRNA candidates were identified both by dRNA-seq and by *in silico* analysis using the SIPHT program (see Discussion).

[b] GEI, genomic islands specific to the K279a genome (33).

[c] That is, the orientations of the upstream gene (column 1), the sRNA (column 2), and the downstream gene (column 3). Strands + and – are indicated by ">" and "<", respectively.

[d] Sequence conservation among bacteria. Abbreviations: K, *S. maltophilia* K279a; R, *S. maltophilia* R551-3; J, *S. maltophilia* JV3; S, *S. maltophilia* SKA14; A, Xanthomonas sp.; B, *Xanthomonas campestris* pv. vesicatoria; C, *Xanthomonas gardneri*; D, *Xanthomonas vesicatoria*; E, *Xanthomonas oryzae*; F, *Xylella fastidiosa*; G, *Pseudomonas* sp.; H, *Pseudomonas aeruginosa*; I, *Acinetobacter baumannii*; L, *Burkholderia multivorans*; M, *Sphingobium japonicum*; N, *Achromobacter xylosoxidans*; O, *Achromobacter piechaudii*; P, *Zymomonas mobilis*.

[e] Same region found upstream of 5 ISStma6.

[f] Same region found upstream of 6 ISStma7.

[g] Same region overlapping 5 ISStma1 by 83 nt.

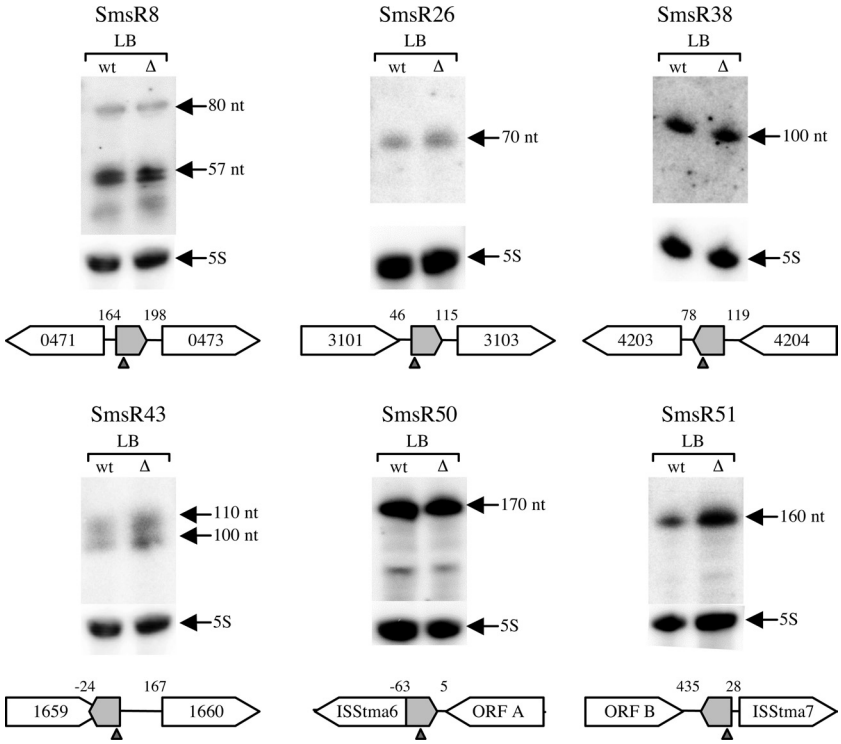

FIG 4 Northern blot analysis of putative *S. maltophilia* sRNAs constitutively expressed. Total RNA was isolated from K279a (wt) and Δ*hfq* (Δ) cells exponentially growing at 37°C in LB medium. The sRNAs indicated at the top were detected using γ^{32}P-labeled complementary oligonucleotides. Major transcripts are marked by arrows. Hybridization signals were quantified with the QuantityOne software package and normalized to signals detected by rehybridization of each filter to an *S. maltophilia* 5S RNA probe. The genomic positions of sRNA genes (gray arrows) relative to adjacent ORFs (white arrows) are shown in the schematic panel (not to scale). The orientation of arrow indicates the transcriptional orientation of sRNA gene and the flanking ORFs. The distances between sRNA gene and flanking ORFs are indicated. Gray triangles indicate the relative position of the probes used. ORF A: Smlt0369, Smlt0465, Smlt2328, Smlt2351, and Smlt2735; ORF B: Smlt0666, Smlt0698, Smlt1093, Smlt1733, Smlt2253, and Smlt3573.

maltophilia may be strictly dependent on specific host factors. In fact, different mouse strains can be either permissive or nonpermissive for the replication of *S. maltophilia* (34).

Based on our dRNA-seq, 63 genes were found to be differentially expressed in wild-type and mutant cells (Table 2) and are potentially regulated directly or indirectly by Hfq. However, our dRNA-seq analysis is only semiquantitative, since it is based on single RNA samples and no biological replicates. Nevertheless, our sequencing data were fully supported by experimental validation of selected target genes by qRT-PCR analyses and monitoring genes downregulated and upregulated in the *hfq* mutant (see Table S3 in the supplemental material).

The defect of the Δ*hfq* strain in cell adhesion and biofilm development is likely correlated to the reduced expression of fimbriae and flagella. The SMF1 (*S. maltophilia* fimbria type 1) protein mediates the adherence of *S. maltophilia* to cultured epithelial cells and plays a role in biofilm formation (8). Flagella are crucial for adhesion of *S. maltophilia* to IB3-1 bronchial cells, swimming motility (31), and biofilm development in the cystic fibrosis isolates (32).

The pleiotropic phenotype of the *E. coli* and *Salmonella hfq* mutants is largely due to interactions of Hfq with regulatory sRNAs (43, 52). Several *S. maltophilia* candidate sRNAs were identified by dRNA-seq (Table 3). Interestingly, half of them (28/60; see Table 3) have been independently identified by computational searches using the program SIPHT (sRNA identification protocol using high-throughput technologies) described by Livny et al. (21). Most of the sRNAs shown in Table 3 are conserved in all *S. maltophilia* strains, whereas some are restricted to K279a, since they are located in genomic islands specific to this strain (33). Interestingly, sRNAs in various bacteria are frequently encoded by genes located within pathogenicity islands and are directly involved in virulence (14, 30). A peculiar class of sRNA is represented by transcripts complementary to the coding region of transposase genes (19). The identification of sRNAs complementary to transposase genes suggests that transposon mobility in *S. maltophilia* might be regulated by an RNA-antisense mechanism, as shown for the Tn*10* transposon (41).

In *E. coli* many sRNAs are protected by Hfq from degradation by RNases (49). The lack of Hfq had no strong effect, at least under the conditions used, on the levels of most of the sRNAs tested, although the binding of one or more sRNAs to Hfq cannot be excluded. *Listeria monocytogenes* LhrB and LhrC sRNAs interact with Hfq, as proven by coimmunoprecipitation assays, but their levels are unaffected by the lack of Hfq (5). The same may hold true for some *S. maltophilia* sRNAs. However, at least the accumulation of two sRNAs, SmsR20 and SmsR36, was severely impaired in the Δ*hfq* mutant, indicating that either the transcription or the stability of these transcripts is Hfq dependent.

Coimmunoprecipitation assays carried out with Flag-tagged Hfq combined with RNAseq (43) should be able to identify all of the sRNAs that directly interact with Hfq, as well as mRNA bind-

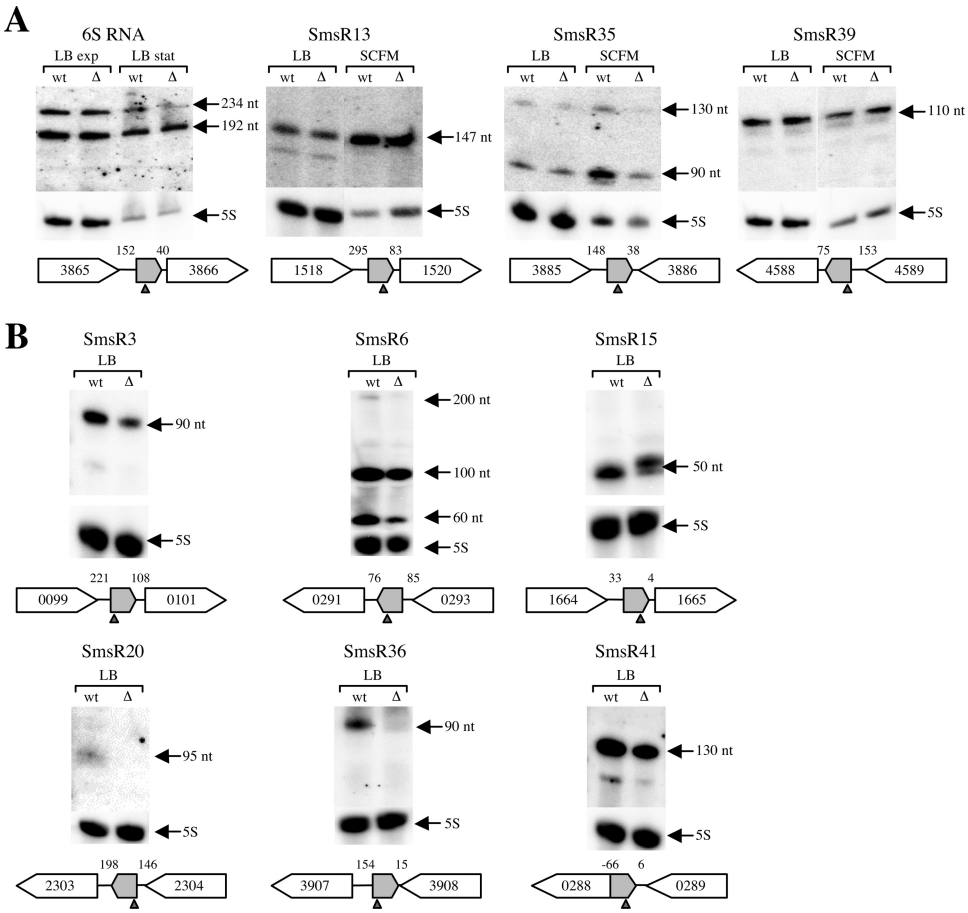

FIG 5 Expression of putative *S. maltophilia* sRNAs depends on growth phase, growth medium, and Hfq. Total RNA was isolated from exponential (exp)- and stationary (stat)-phase cultures of K279a (wt) and Δ*hfq* (Δ) strains growing at 37°C in LB medium or from exponential (exp)-phase cultures of K279a (wt) and Δ*hfq* (Δ) strains growing at 37°C in SCFM. (A) sRNAs that are growth phase and growth medium dependent. (B) sRNAs impaired in the Δ*hfq* mutant. The sRNAs hybridization signals were normalized against 5S RNA as in Fig. 4.

ing partners. The identification of sRNAs interacting with Hfq will identify RNAs and elucidate regulatory circuits that have a role in the pathogenesis of *S. maltophilia*.

ACKNOWLEDGMENTS

This study was supported by a grant assigned to Pier Paolo Di Nocera by the PRIN 2009 agency of the Italian Ministry of University and Scientific Research.

We are indebted to Raffaele Zarrilli for comments and critical reading of the manuscript. We thank Richard Reinhardt (Max Planck Genome Centre, Cologne, Germany) and Jörg Vogel (Institute for Molecular Infection Biology, Würzburg, Germany) for help with deep sequencing.

REFERENCES

1. **Alonso A, Martinez JL.** 1997. Multiple antibiotic resistance in *Stenotrophomonas maltophilia*. Antimicrob. Agents Chemother. **41**:1140–1142.
2. **Anders S, Huber W.** 2010. Differential expression analysis for sequence count data. Genome Biol. **11**:R106.
3. **Brooke JS.** 2012. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. Clin. Microbiol. Rev. **25**:2–41.
4. **Chao Y, Vogel J.** 2010. The role of Hfq in bacterial pathogens. Curr. Opin. Microbiol. **13**:24–33.
5. **Christiansen JK, et al.** 2006. Identification of small Hfq-binding RNAs in *Listeria monocytogenes*. RNA **12**:1383–1396.
6. **Crossman LC, et al.** 2008. The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. Genome Biol. **9**:R74.
7. **De Gregorio E, Silvestro G, Petrillo M, Carlomagno MS, Di Nocera PP.** 2005. Enterobacterial repetitive intergenic consensus sequence repeats in Yersiniae: genomic organization and functional properties. J. Bacteriol. **187**:7945–7954.
8. **de Oliveira-Garcia D, et al.** 2003. Fimbriae and adherence of *Stenotrophomonas maltophilia* to epithelial cells and to abiotic surfaces. Cell Microbiol. **5**:625–636.
9. **De Vidipó LA, De Marques EA, Puchelle E, Plotkowski MC.** 2001. *Stenotrophomonas maltophilia* interaction with human epithelial respiratory cells *in vitro*. Microbiol. Immunol. **45**:563–569.
10. **Di Bonaventura G, et al.** 2010. Role of excessive inflammatory response to *Stenotrophomonas maltophilia* lung infection in DBA/2 mice and implications for cystic fibrosis. Infect. Immun. **78**:2466–2476.
11. **Dietrich M, et al.** 2009. The effect of *hfq* on global gene expression and virulence in *Neisseria gonorrhoeae*. FEBS J. **276**:5507–5520.
12. **Ding Y, Davis BM, Waldor MK.** 2004. Hfq is essential for *Vibrio cholerae* virulence and downregulates sigma expression. Mol. Microbiol. **53**:345–354.
13. **Franze de Fernandez MT, Eoyang L, August JT.** 1968. Factor fraction required for the synthesis of bacteriophage Qβ-RNA. Nature **219**:588–590.
14. **Gong H, et al.** 2011. A Salmonella small non-coding RNA facilitates bacterial invasion and intracellular replication by modulating the expression of virulence factors. PLoS Pathog. **7**:e1002120. doi:10.1371/journal.ppat.1002120.

15. **Hagstrom A, Pinhassi J, Zweifel UL.** 2000. Biogeographical diversity among marine bacterioplankton. Aquat. Microb. Ecol. **21**:231–244.
16. **Hoffmann S, et al.** 2009. Fast mapping of short sequences with mismatches, insertions and deletions using index structures. PLoS Comput. Biol. **5**:e1000502. doi:10.1371/journal.pcbi.1000502.
17. **Jørgensen MG, Nielsen et al.** 2012. Small regulatory RNAs control the multi-cellular adhesive lifestyle of *Escherichia coli*. Mol. Microbiol. **84**: 36–50.
18. **Kulesus RR, Diaz-Perez K, Slechta ES, Eto DS, Mulvey MA.** 2008. Impact of the RNA chaperone Hfq on the fitness and virulence potential of uropathogenic *Escherichia coli*. Infect. Immun. **76**:3019–3026.
19. **Landt SG, et al.** 2008. Small non-coding RNAs in *Caulobacter crescentus*. Mol. Microbiol. **68**:600–614.
20. **Lenz DH, et al.** 2004. The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. Cell **118**:69–82.
21. **Livny J, Teonadi H, Livny M, Waldor MK.** 2008. High-throughput, kingdom-wide prediction and annotation of bacterial non-coding RNAs. PLoS One **3**:e3197. doi:10.1371/journal.pone.0003197.
22. **Logue CA, Peak IR, Beacham IR.** 2009. Facile construction of unmarked deletion mutants in *Burkholderia pseudomallei* using *sacB* counter-selection in sucrose-resistant and sucrose-sensitive isolates. J. Microbiol. Methods **76**:320–323.
23. **Meibom KL, et al.** 2009. Hfq, a novel pleiotropic regulator of virulence-associated genes in *Francisella tularensis*. Infect. Immun. **77**:1866–1880.
24. **Mika F, et al.** 2012. Targeting of csgD by the small regulatory RNA RprA links stationary phase, biofilm formation and cell envelope stress in *Escherichia coli*. Mol. Microbiol. **84**:51–65.
25. **Moller T, et al.** 2002. Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. Mol. Cell **9**:23–30.
26. **Monteiro C, et al.** 2012. Hfq and Hfq-dependent small RNAs are major contributors to multicellular development in *Salmonella enterica* serovar Typhimurium. RNA Biol. [Epub ahead of print.] PMID: 22336758.
27. **Moon K, Gottesman S.** 2009. A PhoQ/P-regulated small RNA regulates sensitivity of *Escherichia coli* to antimicrobial peptides. Mol. Microbiol. **74**:1314–1330.
28. **Nicol JW, Helt GA, Blanchard SG, Jr, Raja A, Loraine AE.** 2009. The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. Bioinformatics **25**:2730–2731.
29. **Palmer KL, Aye LM, Whiteley M.** 2007. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. J. Bacteriol. **189**:8079–8087.
30. **Pichon C, Felden B.** 2005. Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. Proc. Natl. Acad. Sci. U. S. A. **102**:14249–14254.
31. **Pompilio A, et al.** 2010. Adhesion to and biofilm formation on IB3-1 bronchial cells by *Stenotrophomonas maltophilia* isolates from cystic fibrosis patients. BMC Microbiol. **10**:102. doi:10.1186/1471-2180-10-102.
32. **Pompilio A, et al.** 2011. Phenotypic and genotypic characterization of *Stenotrophomonas maltophilia* isolates from patients with cystic fibrosis: genome diversity, biofilm formation, and virulence. BMC Microbiol. **11**: 159. doi:10.1186/1471-2180-11-159.
33. **Rocco F, De Gregorio E, Colonna B, Di Nocera PP.** 2009. *Stenotrophomonas. maltophilia* genomes: a start-up comparison. Int. J. Med. Microbiol. **299**:535–546.
34. **Rouf R, Karaba SM, Dao J, Cianciotto NP.** 2011. *Stenotrophomonas maltophilia* strains replicate and persist in the murine lung, but to significantly different degrees. Microbiology **157**:2133–2142.
35. **Ryan RP, et al.** 2009. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. Nat. Rev. Microbiol. **7**:514–525.
36. **Sanchez P, Moreno E, Martinez JL.** 2005. The biocide triclosan selects *Stenotrophomonas maltophilia* mutants that overproduce the SmeDEF multidrug efflux pump. Antimicrob. Agents Chemother. **49**:781–782.
37. **Sauter C, Basquin J, Suck D.** 2003. Sm-like proteins in Eubacteria: the crystal structure of the Hfq protein from *Escherichia coli*. Nucleic Acids Res. **31**:4091–4098.
38. **Schiano CA, Bellows LE, Lathem WW.** 2010. The small RNA chaperone Hfq is required for the virulence of *Yersinia pseudotuberculosis*. Infect. Immun. **78**:2034–2044.
39. **Seed KD, Dennis JJ.** 2008. Development of *Galleria mellonella* as an alternative infection model for the *Burkholderia cepacia* complex. Infect. Immun. **76**:1267–1275.
40. **Sharma CM, et al.** 2010. The primary transcriptome of the major human pathogen *Helicobacter pylori*. Nature **464**:250–255.
41. **Simons RW, Kleckner N.** 1983. Translational control of IS10 transposition. Cell **34**:683–691.
42. **Sittka A, Pfeiffer V, Tedin K, Vogel J.** 2007. The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium*. Mol. Microbiol. **63**:193–217.
43. **Sittka A, et al.** 2008. Deep sequencing analysis of small noncoding RNA and mRNA targets of the global posttranscriptional regulator, Hfq. PLoS Genet. **4**:e1000163. doi:10.1371/journal.pgen.1000163.
44. **Sonnleitner E, et al.** 2003. Reduced virulence of a *hfq* mutant of *Pseudomonas aeruginosa* O1. Microb. Pathog. **35**:217–228.
45. **Sousa SA, Ramos CG, Moreira LM, Leitão JH.** 2010. The *hfq* gene is required for stress resistance and full virulence of *Burkholderia cepacia* to the nematode *Caenorhabditis elegans*. Microbiology **156**:896–908.
46. **Taghavi S, et al.** 2009. Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Appl. Environ. Microbiol. **75**:748–757.
47. **Thomason MK, Fontaine F, De Lay N, Storz G.** 2012. A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in *Escherichia coli*. Mol. Microbiol. **84**:17–35.
48. **Tsui HC, Leung HC, Winkler ME.** 1994. Characterization of broadly pleiotropic phenotypes caused by an *hfq* insertion mutation in *Escherichia coli* K-12. Mol. Microbiol. **13**:35–49.
49. **Vogel J, Luisi BF.** 2011. Hfq and its constellation of RNA. Nat. Rev. Microbiol. **9**:578–589.
50. **Wassarman KM, Saecker RM.** 2006. Synthesis-mediated release of a small RNA inhibitor of RNA polymerase. Science **314**:1601–1603.
51. **Wu XG, et al.** 2010. Effect of the hfq gene on 2,4-diacetylphloroglucinol production and the PcoI/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24. FEMS Microbiol. Lett. **309**:16–24.
52. **Zhang A, et al.** 2003. Global analysis of small RNA and mRNA targets of Hfq. Mol. Microbiol. **50**:1111–1124.