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The AfaR small RNA controls expression of the AfaD-VIII invasin in pathogenic *Escherichia coli* strains

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ABSTRACT

Pathogenic *Escherichia coli* strains carrying the *afa-8* gene cluster are frequently associated with extra-intestinal infections in humans and animals. The *afa-8* A to E genes determine the formation of an afimbrial adhesive sheath consisting of the AfaD-VIII invasin and the AfaE-VIII adhesin at the bacterial cell surface. This structure is thought to be required for host colonization. We characterized a new gene encoding the small RNA AfaR, which is transcribed in *cis* from the complementary strand of the 3′ untranslated region of the *afaD* messenger RNA, within the *afaD–afaE* intercistronic region. AfaR is a *trans*-acting Hfq-dependent antisense small RNA that binds the 5′ untranslated region of the *afaD* messenger RNA, initiating several ribonuclease E-dependent cleavages, thereby downregulating production of the AfaD-VIII invasin. AfaR transcription is dependent on σ^E, a member of the stress response family of extracytoplasmic alternative sigma factors. We found that the AfaR-dependent regulatory pathway was controlled by temperature, allowing the production of the AfaD-VIII invasin at temperatures above 37°C. Our findings suggest that the entry of *afa-8*-positive pathogenic *E. coli* strains into epithelial cells is tightly regulated by the AfaR small RNA.

INTRODUCTION

Escherichia coli has developed a commensal lifestyle in the lower part of the intestine of humans and other vertebrates, but some strains have the potential to cause a wide spectrum of intestinal and extra-intestinal diseases. Extra-intestinal pathogenic *E. coli* (ExPEC) strains cause bacteraemia, pyelonephritis, cystitis, prostatitis and neonatal meningitis in humans, and various infections in animals. The virulence of ExPEC strains is largely associated with the presence of virulence factors, including adhesins, toxins, siderophores, capsules, invasins and factors contributing to serum resistance (1,2). During the infection process, ExPEC strains must rapidly adapt the expression of their genes in response to both environmental and host signals. A plethora of sensory systems for activating or repressing the expression of virulence genes has thus coevolved with virulence factors.

In regulatory cascades, sensory systems and effectors may be connected by proteins. However, small non-coding RNAs (sRNAs) have recently emerged as a major class of gene regulators of adaptive responses, relaying information from physicochemical stress 'sensing' components to response genes at the post-transcriptional level, without the need for translation (3). A significant proportion of the sRNAs characterized to date interact with dedicated mRNA targets through an antisense-based mechanism, affecting their stability and/or translation (4). Each sRNA is thought to regulate the expression of more than one target, depending on its temporal pattern of expression, thus rendering the system even more complex.

The genome-wide identification of new sRNAs in both gram-negative and gram-positive pathogenic bacteria, by biochemical and *in silico* approaches and, more recently, by high-throughput sequencing analyses, has highlighted the diversity and role of sRNAs (3,5–7). In particular, virulence-associated sRNA genes are abundant in the core genome and pathogenicity islands of the human pathogens *Staphylococcus aureus* (8–11), *Streptococcus agalactiae* (6), *Streptococcus pyogenes* (12), *Salmonella typhimurium* (13,14), *Listeria monocytogenes* (15), *Clostridrium perfringens* (16) and *Vibrio cholerae* (17). However, despite several investigations reporting the identification of new sRNAs in *E. coli*, only a few

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virulence-associated sRNAs from pathogenic strains have been characterized to date. Studies of the role of the Hfq protein, which facilitates gene regulation by sRNAs, showed virulence to be strongly impaired in *hfq*-deficient *E. coli* strains. These strains displayed defects in urinary tract colonization, motility and biofilm production [for uropathogenic *E. coli* UTI89 (18)], together with an impairment of epithelial cell invasion in the case of adherent-invasive *E. coli* LF82 (19). These data suggest that several sRNAs are involved in the control of virulence factor expression by pathogenic *E. coli*/*Shigella* species. However, these molecules have been little studied, other than in quorum sensing (20), *icsA*-dependent invasion of the intestine (21) and type 1-mediated adhesion (6).

During host colonization, ExPEC strains produce several adhesins, including afimbrial adhesins of the Afa family (1,22). All *afa* gene clusters are organized in a similar manner, with six genes (*afaA* to *F*) (1) determining the formation at the cell surface of an adhesive sheath consisting of the AfaD invasin and the AfaE adhesin. These proteins are encoded by the *afaD* and *afaE* genes, and are responsible for bacterial internalization and binding to the host cell, respectively (23–26). Both these structural components are assembled via the chaperone–usher pathway, which is mediated by the proteins encoded by the *afaB* and *afaC* genes (27,28). Finally, the expression of *afa* gene clusters may be controlled by the *afaF* and *afaA* genes, which are homologues of the *papI*/*papB* transcriptional regulator genes (27,29,30). Various subtypes of the *afa* gene cluster (*afa-1* to *afa-8*, *dra*, *daa*) have been described, on the basis of comparisons of the sequences of the *afa* gene cluster, focusing on *afaE* genes in particular (22). The *afa-8* gene cluster is common in pathogenic *afa*-carrying strains isolated from humans and animals (30,31). ExPEC strains carrying *afa-8* gene clusters are frequently detected in humans suffering from pyelonephritis and septicaemia, but these clusters are absent from diarrhoea-associated strains (30). A recent screening for ExPEC-specific sRNA genes showed that some such genes were present in adhesin gene clusters, including the type 1 fimbria operon (6). In particular, a chromosome-borne sRNA candidate gene, *SQ109*, located in the *afa-8* gene cluster of the *E. coli* AL862 strain, has been identified as a putative riboregulator (6).

We show here that SQ109 sRNA downregulates AfaD-VIII invasin production by acting as an Hfq-dependent antisense sRNA, inducing RNase E-dependent *afaD* mRNA decay. The expression of this sRNA gene is dependent on the sigma factor σ^E and temperature, suggesting that AfaD-VIII production is controlled by the environment of the bacterial cell. Based on its location within the *afa-8* gene cluster, we renamed this sRNA gene AfaR.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

The *E. coli* strains and plasmids used in this study are listed in Table 1. All strains were grown in Luria Bertani (LB) broth, at 37°C, with continuous shaking (140 rpm), and were harvested at the indicated OD_{600}. We used the following antibiotics for plasmid selection: 100 μg/ml carbenicillin (Euromedex), 50 μg/ml kanamycin (Sigma), 25 μg/ml chloramphenicol (Sigma), 12.5 μg/ml tetracycline (Sigma) and 100 μg/ml apramycin (Sigma).

General DNA techniques

Oligonucleotides were designed, and *in silico* analysis was carried out with the *afa-8* sequence of *E. coli* 239KH89 (accession number AF072900). The sequences of the oligonucleotides (Eurogentec) used in this study are listed in Supplementary Table S1. All polymerase chain reaction (PCR) products were amplified from the chromosomal DNA of *E. coli* strain 239KH89 (99.7% nucleotide sequence identity to strain AL862). Enzymatic reactions were performed in accordance with the manufacturer's instructions.

Plasmids

We generated the expression plasmid pILL1320 by amplifying the full *afa-8* gene cluster by PCR with the primers clon.afa8.5 and clon.afa8.3 and Taq DNA polymerase (Qbiogen). The PCR product was digested with HindIII (Roche), purified by agarose gel electrophoresis, and ligated into the HindIII site of pBR322.

For expression of the AfaR sRNA, a fragment containing the *afaR* gene without its promoter was amplified by PCR with the Cl.afaR.EcoRI and Cl.afaR.XbaI oligonucleotides. The PCR product was purified by agarose gel electrophoresis and inserted into pCRII-TOPO (Invitrogen). The EcoRI/XbaI fragment of the pCRII-*afaR* plasmid containing the *afaR* gene was purified by agarose gel electrophoresis, ligated between the EcoRI and XbaI sites of pZE2R-*gfp* with T4 DNA ligase (Fermentas) and transferred into the TOP10 strain by electroporation.

We constructed the pZE2R-*afaR** plasmid, encoding the AfaR* mutated sRNA allele, as described for pZE2R-*afaR*. We first introduced two mutations by amplifying the 5′ and 3′ regions of the *afaR* gene with the Cl.afaR.EcoRI/mutafaR5 and mutafaR3/Cl.afaR.XbaI oligonucleotides. The two resulting DNA fragments were assembled by PCR with the Cl.afaR.EcoRI and Cl.afaR.XbaI oligonucleotides.

The pXG*afaD::gfp* fusion plasmid was constructed by amplifying the sequence of the intergenic region including parts of the 3′ end of the *afaC* gene and the 5′ end of the *afaD* gene with the afaC.NsiI and afaD.NheI primers. The PCR product was inserted into pXG-30 digested with NsiI and NheI, as previously described (34). The pXG*afaD*::gfp* fusion plasmid was constructed by introducing mutations into the *afaD* gene by the same strategy used for *afaR**.

The pXG*lacZ::afaD* fusion plasmid was constructed in a similar manner. Briefly, pXG30 was digested with NsiI (Fermentas) and XbaI (Roche), and the 3′ end of the *afaD* gene was amplified by PCR with the afaD.NsiI and afaD.XbaI oligonucleotides, treated with exonuclease I (Fermentas) and digested with XbaI (Roche). The digested plasmid and the PCR product were purified by

Table 1. Strains and plasmids used in this study

Name	Description/relevant characteristics	Phenotype	Source/reference
E. coli strain			
239KH89	Bovine isolate, *afa-8*$^+$		29
AL10	Human clinical isolate, *afa-8*$^+$		31
AL213	Human clinical isolate, *afa-8*$^+$		31
183	Human commensal isolate, *afa-8*$^+$		32
AL511	Human clinical isolate, *afa-8*$^+$		31
AL511Δ*afaR::KmFRT*	*afaR*-deficient AL511 strain		This study
AL862	Human clinical isolate, *afa-8*$^+$		29
N3433	*lacZ43(Fs), LAM-, relA1, spoT1, thi-1*		CGSC# 6976
N3431	N3433 *rne-3071(ts)*		CGSC# 6975
BW25113	K-12 Δ*(araD-araB)567, lacZ4787Δ(::rrnB-3),LAM-, rph-1,* Δ*(rhaD-rhaB),* hsdR514		33
JW4130-1	BW25113 Δ*hfq-722::km*		33
DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ*(lacIZYA-argF)U169 deoR* (ϕ80*lacZ*Δ*M15*) (λ*pyr*$^+$)		Lab. collection
TOP10	*mcr*A Δ*(mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139Δ*(ara-leu)*7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	*Sm*^{R}	34
TOP10Δ*hfq::KmFRT*	*Hfq*-deficient TOP10 strain	*Km*^{R}	34
TOP10Δ*hfq::FRT*	*Hfq*-deficient strain with the FRT-flanked kanamycin cassette removed by flippase-mediated excision with pCP20	*Km*^{S}	This study
MC1061	K-12 F- *araD139* Δ*(ara-leu)7697* Δ*(codB-lacI) galK16 galE15 mcrA0 relA1 rpsL150 spoT1 mcrB9999 hsdR2 lacX74*		Lab. collection
CAG22216	MC1061 [ϕλ*rpoH P3::lacZ*] Δ*rpoE::Cm*	Cm^R	35
Plasmids			
pKD4	Source of the KmFRT resistance cassette	*Km*^{R}	36
pKOBEG-Apra	Derivative of pKOBEG	*Apra*^{R}, *Cm*^{R}	37
pCP20	FLP flippase expression plasmid	*Cb*^{R}	38
pCP22	pCP20 plasmid with insertion of the *ble* gene into XhoI site conferring resistance to zeocin	*Cb*^{R}, *Zeo*^{R}	This study
pBR322	Cloning plasmid	*Cb*^{R}, *Tet*^{R}	Lab. Collection
pILL1320	*afa-8* gene cluster from 239KH89 inserted into HindIII site of pBR322	*Cb*^{R}, *Cm*^{S}	This study
pILL1322	pILL1320 ΔPr-*afaR::FRT*	*Cb*^{R}, *Tet*^{S}	This study
pILL1323	pILL1320 Δ*afaR::FRT*, complete deletion of the region following the rho-independent terminator of *afaD* gene with partial removal of *afaR* gene sequence	*Cb*^{R}, *Tet*^{S}	This study
pILL1324	pILL1320 Δ*afaD::FRT*	*Cb*^{R}, *Tet*^{S}	This study
pILL1325	pILL1322 ΔPr-*afaA::FRT*	*Cb*^{R}, *Tet*^{S}	This study
pZE2R-*gfp*	*gfp* gene under control of the Pλ constitutive promoter	*Km*^{R}	39
pZE2R-null	plasmid encoding a ∼60 nt nonsense RNA	*Km*^{R}	6
pZE2R-*afaR*	*afaR* gene under control of the Pλ constitutive promoter	*Km*^{R}	This study
pZE2R-*afaR**	pZE2R-*afaR* derivative plasmid carrying the GT point mutated allele of AfaR	*Km*^{R}	This study
pQF50	LacZ fusion plasmid	*Cb*^{R}	40
pQF*afaA::lacZ*	5′ UTR of *afaA* inserted into XhoI/XbaI sites of pQF50	*Cb*^{R}	This study
pQF*afaB::lacZ*	5′ UTR of *afaB* inserted into XhoI/XbaI sites of pQF50	*Cb*^{R}	This study
pQF*afaC::lacZ*	5′ UTR of *afaC* inserted into XhoI/XbaI sites of pQF50	*Cb*^{R}	This study
pQF*afaD::lacZ*	5′ UTR of *afaD* inserted into XhoI/XbaI sites of pQF50	*Cb*^{R}	This study
pQF*afaE::lacZ*	5′ UTR of *afaF* inserted into XhoI/XbaI sites of pQF50	*Cb*^{R}	This study
pQF*afaE::lacZ*	5′ UTR of *afaE* inserted into XhoI/XbaI sites of pQF50	*Cb*^{R}	This study
pQF*afaR::lacZ*	5′ region of *afaR* inserted into XhoI/XbaI sites of pQF50	*Cb*^{R}	This study
pXG-0	Translational fusion control plasmid, *gfp*$^-$	*Cm*^{R}	34
pXG-30	Translational fusion plasmid, *gfp*$^+$	*Cm*^{R}	34
pXG*afaD::gfp*	Insertion of the 5′ UTR part of *afaD* into NheI/BfrBI sites of pXG30, giving a *afaD::gfp* translational fusion	*Cm*^{R}	This study
pXG*lacZ ::afaD*	Insertion of the 3′ UTR part of *afaD* into NheI/XbaI sites of pXG30, giving a M2-tagged *lacZ::afaD* translational fusion	*Cm*^{R}	This study
pXG*afaD*::gfp*	pXG*afaD::gfp* derivative plasmid carrying the AC point mutated allele of AfaD	*Cm*^{R}	This study
pACYC184	Cloning plasmid	*Tet*^{R}, *Cm*^{R}	Lab. Collection
pTX381	*hfq* gene inserted into pACYC184	*Tet*^{R}, *Cm*^{R}	41

CGSC, Coli Genetic Stock Center.
Phenotypes: Apra, apramycin; Cb, carbenicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Tet, tetracycline; Zeo, zeocin. S, sensitive; R, resistant.

agarose gel electrophoresis, ligated with T4 DNA ligase (Roche), and introduced into the TOP10 strain by electroporation.

All the promoter fusion plasmids constructed were derivatives of pQF50. The 300 nucleotides upstream from the initiation codon of the *afaA* to *E* coding sequences (CDSs) and the promoter region of *afaR* were amplified by PCR with the primers listed in Supplementary Table S1. PCR products and pQF50 were digested with XhoI and XbaI, purified by agarose gel electrophoresis, ligated with T4 DNA ligase (NEB) and used to transform *E. coli* strain DH5α by electroporation.

All plasmid constructs were sequenced (GATC Biotech) to check that there were no unwanted mutations.

Promoter fusion plasmid assay

We analysed β-galactosidase activity on LB agar plates containing 40 µg/ml X-Gal and 100 µg/ml carbenicilin by scanning bacterial colonies with a PharosFX (Biorad) at an excitation wavelength of 480 nm. The *E. coli* DH5α strain bearing pQF50 was used as a negative control.

Inactivation of genes by allelic exchange

Mutant strains were constructed by the allelic exchange recombination method, with the thermosensitive plasmid pKOBEG-Apra. Briefly, long synthetic primers (~60 nts) overlapping the 5′ and 3′ untranslated regions of target genes were used to amplify the kanamycin FRT-flanked cassette (KmFRT) from pKD4 by PCR. Flippase-mediated excision of the kanamycin resistance cassette was achieved with pCP22.

RNA isolation and reverse transcription

Total RNA was extracted by the hot-phenol protocol (6) and treated twice with DNase I (Roche). We checked the quality of total RNA samples on a Bioanalyzer with an RNA 6000 nanochip (Agilent). Genomic DNA contamination was assessed by PCR amplification of the 5S ribosomal gene with the 5S.Fw and 5S.RT primers, and was considered insignificant if no PCR product was observed on agarose gel electrophoresis. Chimeric DNAs were synthesized from total RNA with random primers for mRNA and specific primers for sRNA, as previously described (6).

Quantitative RT-PCR

For quantitative reverse transcription PCR (qRT-PCR) analyses of gene expression, we used Primer3 software to design specific primer pairs. Real-time PCR was performed with the Bio-Rad iQ system and iQ SYBR Green supermix (Bio-Rad), according to the manufacturer's instructions. The thermocycling protocol was as follows: denaturation at 94°C for 10 min, 40 cycles of amplification and quantification (95°C for 15 s, 60°C for 1 min with a single fluorescence measurement) and a melting curve program (55°C to 95°C, in increments of 0.5°C). Each assay was performed at least in duplicate, with two independently prepared total RNA samples. The relative expression data obtained were analysed with the optimized amplification efficiency protocol derived from the ΔΔCt method, with the 5S gene used as a reference gene (42).

5′ and circular RACE

The 5′ RACE experiments were carried out as previously described, but with the following modifications (43). Briefly, 10 µg of freshly prepared total RNA from the 239KH89 strain was left untreated or treated with the TAP enzyme (Epicentre) and ligated with the R1 RNA adapter (Sigma Proligo). Chimeric DNA was synthesized with Superscript III reverse transcriptase (Invitrogen) and random primers (Invitrogen). It was then amplified by PCR with P10 and reverse primers and analysed by agarose gel electrophoresis. The largest TAP-treated PCR products were extracted from the gel, inserted into pCRII-TOPO (Invitrogen) and sequenced (GATC Biotech).

Circular RACE was carried out on freshly prepared total RNA. The RNA was first denatured by heating at 80°C for 2 min in 1× buffer (10 mM Tris-HCl pH 8.1 at 37°C, 7% deionized formamide, 0.2% SDS) and was then dephosphorylated with CIP enzyme (NEB). The RNA was purified by phenol/chloroform extraction and isopropanol precipitation. The 5′ ends of RNAs were re-phosphorylated with T4 polynucleotide kinase (NEB) in 1× buffer (80 mM Tris-HCl pH 8.1 at 37°C, 8 mM $MgCl_2$, 4 mM dithiothreitol, 1 mM spermidine), 1 mM ATP and 40 units of RNaseOUT (Invitrogen). The RNA sample was self-ligated, with or without T4 RNA ligase (Epicentre). Circular RNA products were reverse transcribed with random primers, and the products were amplified by PCR with rigBC.RT and rigCD.Fw primers and checked by agarose gel electrophoresis. The DNA of bands of interest (detected only after the use of T4 RNA ligase) was extracted from the agarose gel with the Qiagen gel extraction kit, inserted into pCRII-TOPO and sequenced.

Measurement of RNA decay by rifampicin assay

We measured the rate of decay of AfaR sRNA by growing strain carrying the pZE2R-*afaR* plasmid in LB broth, at 37°C or 42°C, with continuous shaking (140 rpm) until the OD_{600} reached 0.4. Rifampicin was added to stop the initiation of transcription (final concentration, 500 µg/ml). Aliquots of cells were removed at several time points (0, 1, 2, 4, 8, 16, 30 and 60 min) after the addition of rifampicin and immediately frozen by mixing with an equal volume of cooled absolute ethanol. RNA preparation, cDNA synthesis and quantitative RT-PCR were performed as described above. The half-life was calculated from the slope of a least-squares regression line of a semi-logarithmic plot of the percentage sRNA remaining as a function of time. Half-life was calculated from the time points at which decay rate was exponential.

Western blotting

Crude extracts of bacterial cell were separated by SDS-PAGE in 14% polyacrylamide gels, and the protein bands were electro-transferred onto PVDF

membranes (Millipore). The membranes were then hybridized with the α-GFP antibody (Roche), the α-FLAG antibody (Sigma-Aldrich) and α-GroEL antisera (Sigma-Aldrich) as previously described (34). The AfaD-VIII and AfaE-VIII proteins were detected by incubation with rabbit polyclonal anti-rAfaD-VIII or anti-rAfaE-VIII antibodies (25) at a dilution of 1:2000, followed by horseradish peroxidase (HRP)-conjugated anti-rabbit serum (Zymed). The membranes were then developed with the SuperSignal West pico chemiluminescent reagent (Pierce), and the signals were detected with a ChemiDoc XRS system (Biorad).

RESULTS

Transcriptional organization of the *afa-8* gene cluster

The sequence and genetic organization of the *afa-8* gene cluster of *E. coli* 239KH89 strain were described a few years ago, but we still know little about its transcription (29). We first predicted the transcriptional units of this *afa-8* gene cluster *in silico* by determining the location of the promoters and Rho-independent terminators. Putative σ^{70} promoters were identified upstream from the *afaA*, *afaE* and *afaF* genes (Figure 1A). These promoters were validated by fusing the 300-bp DNA fragment upstream from the AUG start codon of each *afa* CDS with the *lacZ* reporter gene of the pQF50 plasmid. β-galactosidase activity was observed only for strains carrying pQF*afaA::lacZ*, pQF*afaE::lacZ* and pQF*afaF::lacZ* (Figure 1B), confirming our predictions. The identification of a Rho-independent terminator beginning 240 bp downstream from the stop codon of the *afaD* gene suggests that the *afaD* mRNA has a long 3′ UTR. Another Rho-independent terminator was found 25 bp downstream from the *afaE* stop codon. In a previous study, the ~230-nt SQ109 sRNA gene was identified between the *afaD* and the *afaE* genes (6). This sRNA was renamed AfaR (afimbrial adhesin small RNA), as its location suggested a probable role in regulating the expression of the *afa-8* gene cluster. BLASTN analysis against the GenBank database (24 January 2012) revealed that the sequence and synteny of the *afaR* gene were conserved in all sequenced *afa-8* gene clusters from *E. coli* isolates (239KH89, AL511 and AL862 strains) but that this gene was absent from all other *afa* family gene clusters. We mapped the transcription start site of *afaR* by 5′ RACE to nucleotide position A_{5534} of the *afa-8* gene cluster of

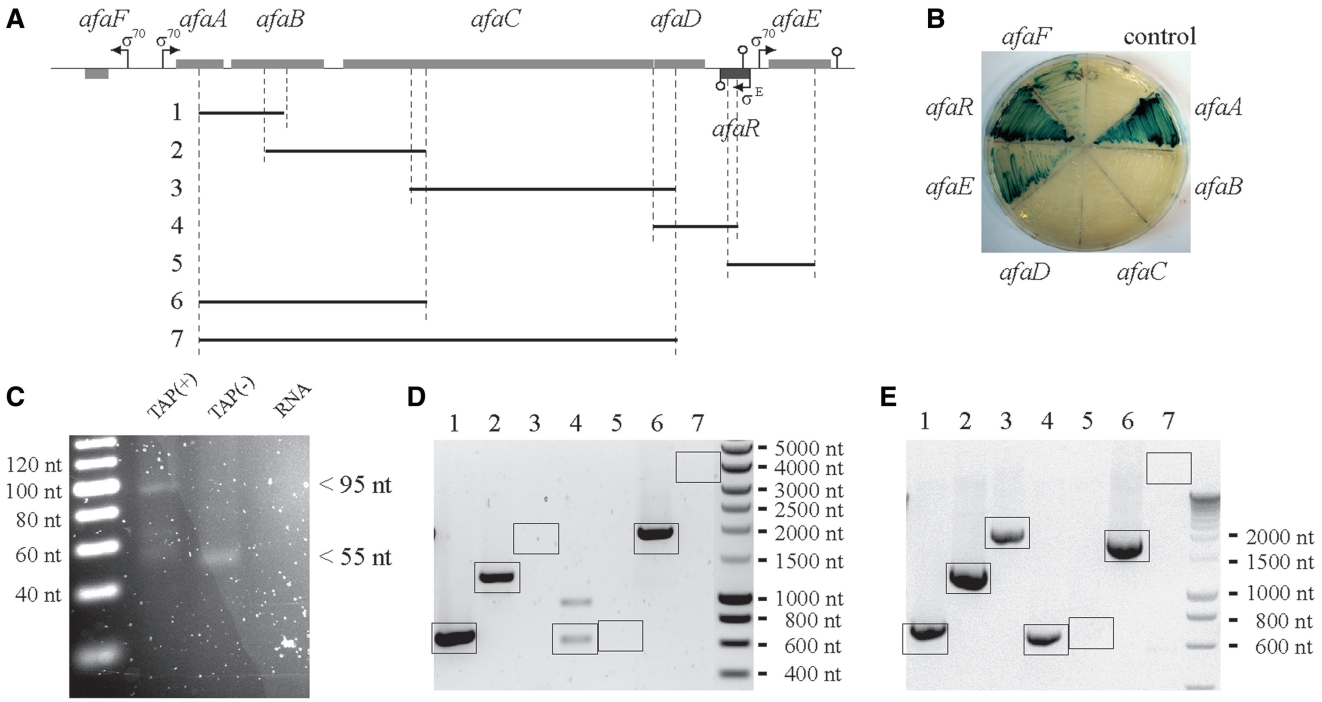

Figure 1. Organization of the transcriptional units of the *afa-8* gene cluster from *E. coli* 239KH89. (**A**) Genetic organization of the *afa-8* gene cluster, with predicted promoters (arrows) and rho-independent terminators (circles). Position of primers (vertical dashed line) for PCR products [horizontal black lines 1 to 7 refer to the RT-PCR shown in (D) and (E)]. (**B**) β-Galactosidase plate activity assays. Each MC1061 strain carries a pQF50-derived plasmid with or without the region upstream from an *afa* gene fused to *lacZ*. (**C**) 5′ RACE for identification of the AfaR transcription start site. Total RNA from *E. coli* strain 239KH89 was linked to a 5′ RNA adaptor with [TAP (+) lane] or without [TAP (−) lane] removal of the 5′ triphosphate by TAP treatment. After RT-PCR amplification with specific primers, PCR products were analysed by electrophoresis in 4% agarose gels. A single ~95 bp band strongly enhanced by TAP treatment was cloned and sequenced to identify the transcription start site of the *afaR* gene. (**D, E**) Cotranscription analysis of the *afaA* to *E* genes from the *afa-8* cluster. (D) Analysis of the BW25113 + pILL1320 strain. (E) Analysis of the BW25113 + pILL1322 strain. The products of RT-PCR amplification were analysed by electrophoresis in a 1% agarose gel, which demonstrated probable linkage between *afaA* and *afaB* (lane 1), *afaB* and *afaC* (lane 2), *afaC* and *afaD* (lane 3), *afaD* and the 3′ UTR of *afaD* (lane 4), the 3′ UTR of *afaD* and *afaE* (lane 5), *afaA* and *afaC* (lane 6), *afaA* and *afaD* (lane7). The expected PCR products are indicated by boxes.

E. coli 239KH89 (Figure 1C). An analysis of the *afaR* promoter region showed strong sequence identity to the consensus sequence of the σ^E (also named σ^{24}) promoter. In particular, this region contains the 'AA' tract of the -35 element, the invariable C-residue of the -10 box, the 16-bp spacer, the 6-bp discriminator sequence and the conserved -1 T-residue (44). The AfaR promoter was validated by constructing the pQF*afaR::lacZ* fusion plasmid, which yielded β-galactosidase expression (Figure 1B). The distance between the σ^E promoter and the Rho-independent terminator confirmed the previous estimate of 230 nt for the size of the AfaR sRNA (6).

Owing to its antibiotic multiresistance phenotype, genetic modification of *E. coli* strain 239KH89 was not possible. We therefore carried out most of the experiments on the *E. coli* BW25113 laboratory strain carrying pILL1320, corresponding to the entire *afa-8* gene cluster inserted into the pBR322 plasmid. Several RT-PCRs were performed to determine whether the *afaABCDE* genes were cotranscribed in the BW25113 + pILL1320 strain; for these reactions, we used primers targeting the 5′ and 3′ ends of each gene and the 3′ UTR of *afaD* (Figure 1A and D). The specificity of the reverse transcription reactions was demonstrated in control experiments by omitting the reverse transcriptase enzyme (data not shown). We checked that the transcription profile of the *afaABCDE* genes was similar in the *E. coli* 239KH89 and BW25113 + pILL1320 strains (Supplementary Figure S1). Our findings suggested that *afaA* and *afaB*, and *afaB* and *afaC* were cotranscribed, generating a single mRNA molecule. However, no linkage was detected between *afaC* and *afaD*, *afaD* and *afaE* or between *afaA* and *afaD*. Amplification of the *afaD* and *afaD* 3′ UTR mRNAs but not of the *afaD* 3′ UTR and *afaE* mRNAs confirmed the location of the Rho-independent terminator and suggested that *afaE* was independently transcribed. qRT-PCR analysis of the transcription of the *afa-8* gene cluster of the BW25113 + pILL1320 strain revealed that relative transcript levels for the *afaAB*, *afaC* and *afaD* units were significantly different (Supplementary Figure S1). We interpreted these observations as indicating that a putative *afaABCD* mRNA could be cleaved by one or more endoribonucleases (RNases) at sites surrounding the *afaC* mRNA, resulting in the release of the *afaD* mRNA. The cotranscription of the *afaABCD* mRNA did not rule out the presence of a putative internal promoter. We therefore deleted the entire promoter region between *afaF* and *afaA* and the region downstream from the *afaD* Rho-independent terminator, including the *afaR* gene promoter from the pill1320 plasmid, giving pILL1325. Analysis, by qRT-PCR, of the transcription of the *afa-8* cluster genes from the BW25131 + pILL1325 revealed no significant amplification of *afaABCD* genes with respect to the 5S gene, indicating that there was not such internal promoter in the gene cluster. In addition to, the *afaE* gene was amplified, confirming that it was an independent gene (data not shown). Taken together, these results suggest that the *afa-8* gene cluster consists of four transcriptional units: *afaABCD*, *afaR*, *afaE* and *afaF*.

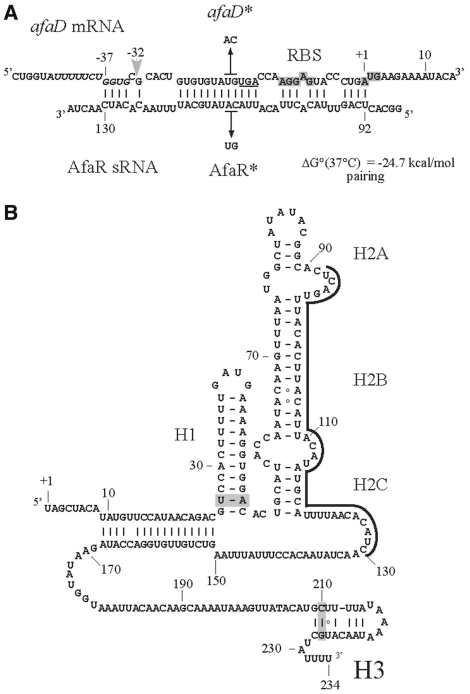

Figure 2. Predicted RNA secondary structures. (**A**) Nucleotide pairing of AfaR with the 5′ UTR of the *afaD* mRNA from *E. coli* 239KH89. The putative RBS required for *afaD* translation and the ATG codon are highlighted in grey. Numbers denote the residues in the *afaD* mRNA with respect to the ATG codon and the mapped +1 site of AfaR. The positions of point mutations introduced into the *afaD* gene (TG_{4803} to AC, giving the *afaD** allele) and AfaR (AC_{110} to TG, giving the AfaR* allele) and expected to maintain base-pairing between the *afaD**/AfaR* duplex, are indicated by thin black arrows. The light grey arrow indicates the putative RNase E cleavage site at G_{4791} (-32 relative to the ATG codon). The AU-rich sequence next to a stem–loop helix and a G residue positioned two nucleotides upstream from a cleavage site mimicking the RNase E recognition sequence are displayed in italics. The G residue is shown in italics and underlined. The stop codon of the *afaC* gene is underlined. (**B**) Predicted secondary structures of AfaR sRNA. The region of the *afaD* mRNA to which AfaR binds is underlined. Covariations supporting structure prediction are highlighted in grey.

AfaR interacts with the *afaD* mRNA via an antisense mechanism

Our working hypothesis is that AfaR is an antisense RNA that regulates AfaD production. We therefore used intaRNA software (45) to analyse *in silico* the likelihood of AfaR forming double-stranded antisense pairs with other *afa-8* gene mRNAs. One significant putative antisense pairing was predicted between nucleotides U_{92} to C_{130} of AfaR and nucleotides G_{-37} to A_{+1} of the *afaD* mRNA 5′ UTR, which contains the ribosome-binding site (RBS) (Figure 2A). A second predicted pairing involved the 3′ UTR of the *afaD* mRNA (residues T_{490} to G_{695}), which perfectly matched the last 206 nt of the AfaR sequence (data not shown). This putative pairing was predicted on the basis of the presence of *afaD* and the 3′ end of the *afaR* gene in the same DNA locus, but in opposite orientations. Both pairing regions were conserved in *E. coli* strains carrying *afa-8* gene clusters, as were AfaR secondary structures (Figure 2B). We thus hypothesized that AfaR bound the 5′ and/or 3′ UTR of the *afaD* mRNA, thereby modulating its translation and/or stability.

For the validation of these predictions *in vivo*, we assessed the putative base-pairing interaction between AfaR and these two mRNA targets, using a translational control and target recognition system (34). The *afaD* 5′ region, spanning the last 66 bp of the *afaC* gene and the first 60 bp of the *afaD* gene, was inserted into pXG-30, giving pXG*afaD::gfp*. The expression of the *afaD::gfp* translational fusion gene was under the control of the $P_{LtetO-1}$ promoter. The *afaR* gene was placed under the control of the strong constitutive P_{λ} promoter in the pZE2R-*afaR* plasmid. Four *E. coli* TOP10 strains harbouring a combination of the pXG*afaD::gfp* target plasmid or pXG-0 (no target control) and either pZE2R-*afaR* or pZE2R-null were constructed. We monitored the levels of the sRNA and fusion mRNAs by qRT-PCR and GFP production by Western blotting (Figure 3A). AfaR over-expression was associated with a decrease in the amount of the *afaD::gfp* fusion mRNA by a factor of 2 to 2.56 (Figure 3A). Interestingly, AfaR levels decreased with those of the *afaD* mRNA target, suggesting AfaR-guided ribonuclease degradation of the target. Western blot experiments with antibodies directed against GFP showed that AfaR overexpression was associated with a halving of AfaD::GFP protein levels, consistent with the results of the transcriptional analysis (Figure 3A).

The AfaR/*afaD* RNA interaction was validated by introducing point mutations into the pairing regions of the AfaR gene and the *afaD* mRNA 5′ UTR (Figure 2A). The TG_{-19} dinucleotide (UG in RNA) of *afaD* was changed to AC, yielding the *afaD*::gfp* allele, and the CA_{109} dinucleotide in *afaR* was changed to GT, giving a compensatory *afaR** allele. Western blot analyses showed that regulation of the *afaD::gfp* fusion by AfaR* was strongly impaired. Similarly, the *afaD*::gfp* fusion was more resistant to regulation by wild-type AfaR allele expression from pZE2R-*afaR*. The regulation of *afaD*::gfp* fusion expression was restored by expressing the compensatory AfaR* allele from pZE2R-*afaR** (Figure 3B).

The 3′ UTR of the *afaD* mRNA was the second putative target of AfaR determined *in silico*. We investigated whether the *afaD* 3′ UTR played a critical role in regulation by inserting the sequence spanning the last 60 bp of the *afaD* gene and the following 278 bp sequence, including the *afaD* rho-independent terminator, into pXG30, to obtain pXG*lacZ::afaD*. Expression of the *lacZ::afaD* fusion was monitored by qRT-PCR and Western blotting in *E. coli* TOP10 harbouring a combination of the pXG*lacZ::afaD* target plasmid or pXG-0 (control containing no target sequence) and either pZE2R-*afaR* or pZE2R-null (Figure 3C). A comparison of the relative levels of *lacZ::afaD* mRNA in strains bearing pZE2R-*afaR* or pZE2R-null showed that AfaR overexpression had no effect on fusion mRNA levels (Figure 3C). Western blots with antibodies directed against the M2-FLAG tag (LacZ) confirmed this observation (Figure 3C). Thus, AfaR is an antisense sRNA that represses *afaD* expression *in vivo* by interacting with the 5′ UTR of the *afaD* mRNA but not with its 3′ UTR.

AfaR controls *afaD* mRNA levels posttranscriptionally

We constructed an *afaR* mutant strain with a view to confirming the target of AfaR *in vivo*. *E. coli* strain 239KH89 is resistant to several antibiotics, AL511 does not express *afaE* (Supplementary Figure S1) and AL862 carries two *afa-8* gene clusters (29). We therefore mutated the sequence inserted into pILL1320. As the 3′ sequences of the *afaD* and *afaR* genes overlapped, we had to delete the σ^E promoter of *afaR* to abolish the expression of this gene, to generate pILL1322 (Figure 4A). We checked that the *afaR* sequence in the pILL1322 plasmid was not expressed by qRT-PCR (Figure 4B). A transcriptional linkage analysis of BW25113 + pILL1322 showed that *afaC* and *afaD* were cotranscribed. This suggested that AfaR might be involved in the release of *afaD* mRNA from a primary transcript (Figure 1E), consistent with an interaction between the two RNAs based on an antisense mechanism. We then determined the relative levels of expression of the *afaABCDE* genes in BW25113 + pILL1320 and BW25113 + pILL1322 by qRT-PCR (Figure 4B). RNA was extracted from cultures at an OD_{600} of 0.6, in LB medium, in which AfaR levels were maximal (data not shown). No significant difference was observed in the relative levels of expression of *afaABC* and *afaE*, indicating that AfaR had little transcriptional or posttranscriptional effect on the expression of these genes.

However, the inhibition of AfaR increased *afaD* expression by a factor of 3.8 (Figure 4B), and Western blotting indicated an accumulation of the AfaD protein (~15 kDa) by a factor of 2.6 in the BW25113 + pILL1322 strain (Figure 4C). We validated these results by constructing the AL511 Δ*afaR::KmFRT* strain and analysing the expression of the *afa-8* gene cluster of this mutant by qRT-PCR, comparing the results obtained with those for the wild-type AL511 strain. The transcription levels of the *afaA*, *B* and *C* genes were similar in the two strains. By contrast, the *afaD* mRNA was overproduced in the *afaR*-lacking AL511 strain, as previously reported for BW25113 + pILL1322 (Figure 4D). Taken together, these results suggest that AfaD expression is controlled posttranscriptionally by AfaR.

The pairing of the *afaR* and *afaD* mRNAs is dependent on Hfq

In *E. coli*, most antisense sRNAs make use of the Hfq protein to capture their mRNA targets (46). We assessed the contribution of Hfq to the AfaR-mediated regulation of *afaD* mRNA *in vivo* by comparing the levels of *afaD* transcripts in the *hfq*-depleted strain JW4130-1 and the isogenic BW25113 strain, both carrying pILL1320. The relative level of *afaA* mRNA in the *hfq* mutant was one tenth that in the wild type, as shown by qRT-PCR, indicating that Hfq affected the transcription of the *afa-8* gene cluster (data not shown). When the pTX381 plasmid (carrying the *E. coli hfq* gene) was introduced into the JW4130-1 + pILL1320 strain, *afaA* mRNA levels were restored to wild-type values (data not shown). We therefore normalized gene expression with respect to *afaA* in qRT-PCR analyses, to evaluate the

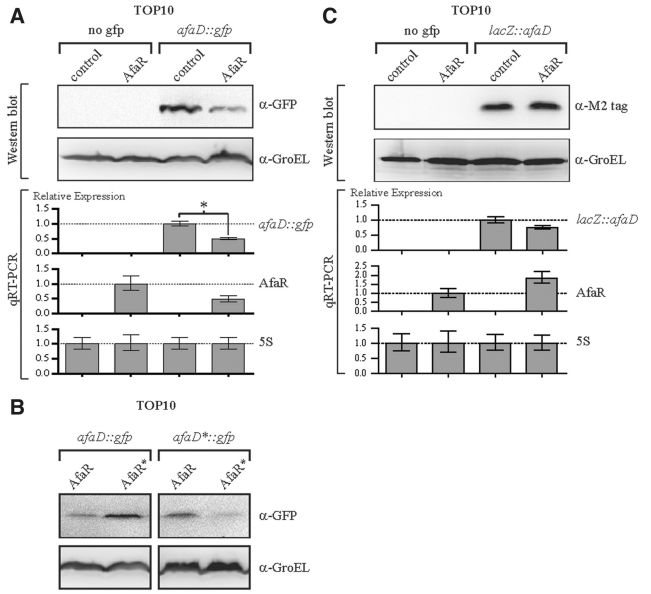

Figure 3. AfaR-induced *afaD* mRNA decay occurred owing to the binding of AfaR to the 5′ UTR of the *afaD* mRNA. (**A**) Analysis of binding to the 5′ UTR of *afaD* mRNA. Analysis, by qRT-PCR and Western blotting, of *afaD::gfp* and AfaR gene expression in *E. coli* strain TOP10 harbouring pZE2R-*afaR* or pZE2R-null together with pXG-0 (no target control) or pXG*afaD::gfp* target expression plasmids. The four isolates were cultured in LB medium at 37°C, to an OD_{600} of 1.0. The expression of the *gfp* fusion gene was normalized to 1.0 for the TOP10 vector carrying pZE2R-null and pXG*afaD::gfp*. AfaR expression was normalized to 1.0 for TOP10 carrying pZE2R-*afaR* and pXG-0. The GroEL protein was used as a loading control. (**B**) Western-blot analysis with antibodies directed against GFP, for TOP10 harbouring pZE2R-*afaR* or mutated pZE2R-*afaR** together with either wild-type pXG*afaD::gfp* or mutant pXG*afaD*::gfp* fusion plasmids. (**C**) Analysis of binding to the 3′ UTR of *afaD* mRNA. qRT-PCR and Western blot analysis were performed as described in (A), but with strains carrying pXG*lacZ::afaD* instead of the pXG*afaD::gfp* fusion.

transcription-independent effects of Hfq (Figure 5A). The deletion of *hfq* had a significant effect on the relative levels of *afaC* and *afaD* mRNAs and AfaR (Figure 5A), suggesting possible cooperation between AfaR and Hfq. We tested this hypothesis by carrying out the same experiment with the JW4130-1 + pILL1322 strain, in which expression levels were similar to those for JW4130-1 + pILL1320 (Figure 5B). We characterized this effect in more detail by transforming the TOP10 Δ*hfq* strain harbouring either pZE2R-*afaR* or pZE2R-null with pXG*afaD::gfp* and monitoring the expression of the *afaD::gfp* fusions and AfaR by qRT-PCR. The overtranscription of AfaR did not significantly decrease the amount of fusion mRNA. Furthermore, AfaD::GFP fusion protein levels were slightly higher in the presence of the AfaR sRNA, as shown by Western blots with α-GFP serum. The spot intensities for the fusion proteins in each lane were normalized with respect to the GroEL loading control for the lane concerned. The differences in normalized spot intensities were not statistically significant (*t*-test) (Figure 5C). We infer from these findings that the Hfq protein is required for the AfaR-mediated regulation of *afaD* expression.

AfaR and RNase E-dependent cleavages of *afaD* mRNA

Characterization of the transcriptional units of the *afa-8* gene cluster suggested a possible role for RNases in the posttranscriptional regulation of *afaABCD* mRNA (Figure 1C and D). We tested this hypothesis by analysing the dependence of *afa-8* cluster gene expression on RNase E, an RNase frequently associated with sRNA mechanisms. We introduced pILL1320 into the N3431 strain carrying the thermosensitive allele *rne*-3071 (encoding $RNaseE^{ts}$) and the isogenic strain (N3433) carrying the wild-type *rne* gene (encoding RNase E). Both strains were grown to an OD_{600} of 0.6 at 30°C, and half the culture was then shifted to 42°C for 30 min to inactivate rne^{ts} gene expression. We analysed the relative levels of *afaA* and *afaD* mRNAs and of AfaR in the two strains by qRT-PCR. In the control RNase E^+ strain context (Figure 6C), *afaA* expression levels were unaffecting, indicating that a shift in temperature to 42°C had no visible effect on transcription of the *afaABCD* operon. By contrast, this shift had a strong effect on AfaR levels. When the rne^{ts} gene was inactivated, we observed a small, but marginally significant, increase by a factor of 2.4 in the relative level of the *afaA* transcript (Figure 6B). By contrast, the levels of the *afaD* and AfaR RNAs were 4.6 and 16.8 times higher, respectively (Figure 6B), indicating a possible role of RNase E in the posttranscriptional processing of *afaD* mRNA. A similar analysis was carried out with the RNase E^{ts} strain carrying the pILL1322 plasmid. The levels of the *afaA* and *afaD* mRNAs did not change significantly with temperature, indicating that AfaR was required for RNase E-dependent cleavage of the *afaABCD* mRNA (Figure 6D).

We assessed the possible AfaR-dependent cleavage of the *afaABCD* mRNA by carrying out differential circular RACE experiments between strains BW25113 carrying pILL1320 or pILL1323, a derivative of pILL1320 containing only the complete *afaABCD* operon with its natural rho-independent terminator (Figure 6A). The use of this construct overcomes problems due to the influence of downstream sequences on the non-specific expression of AfaR. RNase cleavage occurred around *afaC*. We therefore grew both isolates to an OD_{600} of 0.6, extracted total RNA and analysed both AfaR levels and the ligation-dependent circularization of the *afaC* mRNA (See Methods section, Figure 6E). We sequenced 20 recombinant plasmids containing the ligated 5′ and 3′ ends of the *afaC* mRNA (Supplementary Figure S2). We filtered out sequences that were not identified at least three times, to distinguish between AfaR-dependent RNase activity and non-specific cleavage. Three cleavage sites were identified at positions U_{-2780}, G_{-32} and A_{66}, within the 5′ end of the *afaC* mRNA, the 5′ end of the *afaD* mRNA that paired with AfaR and within the coding region of the *afaD* mRNA, respectively (Figure 4A and 6E). Relative levels of *afaB* and *afaC* mRNA in BW25113 + pILL1322 and BW25113 + pILL1320 were similar, indicating that cleavage in the 5′ UTR region of the *afaC* mRNA was independent of AfaR (Figure 4B). For confirmation that *afaD* mRNA cleavage was dependent on AfaR, we performed a differential 5′ RACE experiment on

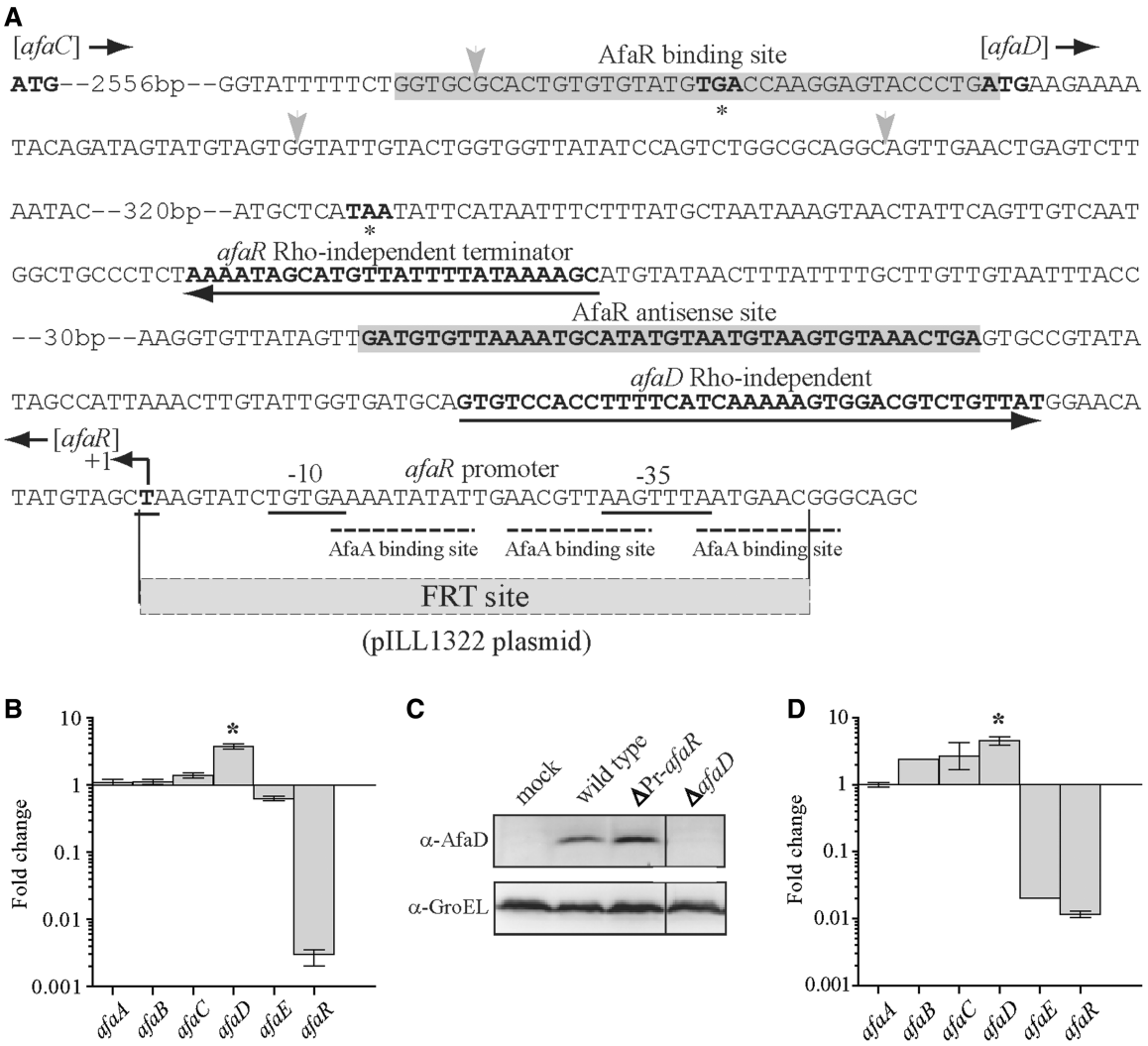

Figure 4. Posttranscriptional control of *afaD* mRNA levels by AfaR, *in vivo*. (**A**) Overview of the *afaD*/*afaR* locus from the *afa-8* gene cluster of *E. coli* strain 239KH89. The promoter boxes (-10 and -35) and transcription start site (+1) of the *afaR* gene are underlined. Rho-independent terminators are indicated by an arrow. 'ATG' in bold and asterisks indicate the initiation and termination codons, respectively, of the *afaC* and *afaD* CDSs. The grey box indicates the binding region of the AfaR sRNA and *afaD* mRNA. Vertical grey arrows indicate the position of ribonuclease cleavages. The pILL1322 plasmid has the same sequence as pILL1320, except that the σ^E promoter sequence is replaced by the FRT site, as indicated. (**B**) Assessment of the relative expression of the *afaABCDER* genes in strain BW25113 carrying pILL1322 (ΔPr-*afaR*) versus pILL1320 (wild type), determined by qRT-PCR. Bacteria were grown to an OD_{600} of 0.6. (**C**) Western blot analysis (with an anti-rAfaD-VIII antiserum) of AfaD production in a total protein extract obtained at an OD_{600} of 0.6 from BW25115 carrying pBR322 (mock), pILL1320 (wild type), pILL1322 (ΔPr-*afaR*) or pILL1324 (Δ*afaD*). The GroEL protein was used as a loading control. (**D**) Analysis, by qRT-PCR of the relative expression of the *afaABCDER* genes from the AL511 Δ*afaR::KmFRT* and AL511 strains grown to an OD_{600} of 0.6. Note: the *afaE* gene was naturally not expressed in the *E. coli* AL511 wild-type strain.

afaD mRNA in the BW25113 + pILL1320 and BW25113 + pILL1323 strains (data not shown). The AfaR-dependent cleavage site at position A_{66} within the *afaD* mRNA was confirmed, and an additional cleavage site was revealed at position G_{29} of the *afaD* mRNA (Figure 6F).

RNA secondary structure prediction close to the RBS of *afaD* (Figure 2A) showed an AU-rich sequence next to a stem–loop helix and a G residue positioned two nucleotides upstream from a cleavage site mimicking the RNase E recognition sequence (47). Thus, cleavage in the 5′ UTR and, probably, within the coding region of the *afaD* mRNA were dependent on RNase E.

The expression of the *afaR* gene is controlled by temperature and a σ^E promoter

The AfaR gene may be transcribed by the alternative σ^E, a sigma factor associated with the response to extracytoplasmic stresses, such as increasing temperature. We therefore assessed dependence on the σ^E promoter by introducing the pQF*afaR::lacZ* plasmid into *E. coli* wild-type and isogenic Δ*rpoE* mutant strains and assessing its activity by measuring β-galactosidase activity (Figure 7A and B). The *afaR* promoter had no transcriptional activity in the Δ*rpoE* strain, in either the exponential or the stationary phase. A comparison of *afaR::lacZ* fusion mRNA levels in the two strains confirmed these

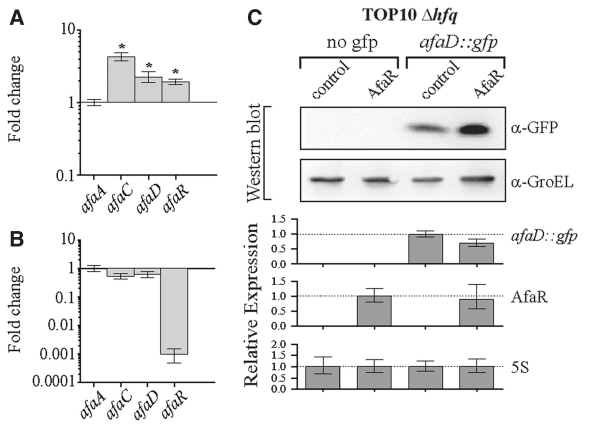

Figure 5. Expression of the *hfq* gene was required for the AfaR-dependent regulation of *afaD* mRNA decay. (**A**) Assessment of the relative expression of the genes of the *afa-8* cluster in strain JW4130-1 (Δ*hfq*) carrying pILL1320 versus BW25113 carrying pILL1320, determined by qRT-PCR. Asterisks indicate significant differences. (**B**) Assessment of the relative expression of the genes of the *afa-8* cluster in strain JW4130-1 (Δ*hfq*) carrying pILL1322 versus JW4130-1 carrying pILL1320, determined by qRT-PCR. (**C**) Analysis by Western blotting and qRT-PCR of *afaD::gfp* and AfaR gene expression in *E. coli* strain TOP10 Δ*hfq::FRT* harbouring pZE2R-*afaR* or pZE2R-null plasmids together with pXG-0 (no target control) or pXG*afaD::gfp* target expression plasmids. The four isolates were cultured in LB medium at 37°C, to an OD_{600} of 1.0. Expression levels of the *gfp* fusion gene were normalized to 1.0 for TOP10 Δ*hfq::FRT* carrying pZE2R-null and pXG*afaD::gfp*. AfaR expression was normalized to 1.0 for TOP10 Δ*hfq::FRT* carrying pZE2R-*afaR* and pXG-0. GroEL was used as a loading control.

findings (Figure 7C). Thus, AfaR transcription is dependent on σ^E.

Previous experiments had indicated that expression of the *afa-8* gene cluster was temperature dependent and suggested that the σ^E-dependent AfaR sRNA was responsible for the thermoregulation of AfaD production (C. Le Bouguénec, personal communication). This hypothesis was tested by growing the BW25113 + pILL1320 strain at 20°C, 30°C, 37°C and 42°C, to an OD_{600} of 0.6. The relative levels of the *afaA* and *afaD* mRNAs and AfaR were analysed by qRT-PCR (Figure 8A), and AfaD production was assessed by Western blotting (Figure 8B). AfaR levels were higher at 37°C than at any other temperature. However, although *afaD* mRNA levels were high at 37°C, the level of AfaD production was lower, indicating that temperature controlled AfaR expression and the fine regulation of AfaD production. We tested this hypothesis by performing the same experiments with the BW25113 + pILL1323 strain. We found that knockout of the *afaR* gene led to the constitutive production of *afaD* mRNA and AfaD (Figure 8C and D).

The abundance of an sRNA at a given time point is a reflection of its rate of synthesis, mode of regulation and decay. We assessed the stability of the AfaR sRNAs by performing qRT-PCR analysis with total RNA isolated from the BW25113 + pZE2R-*afaR* strain cultured at 37°C and 42°C to an OD_{600} of 0.4 before treatment with rifampicin to inhibit RNA synthesis. The half-life of AfaR was found to be 6.9 min ± 21 s at 37°C and 5.04 min ± 17.7 s at 42°C. Given that the AfaR sRNA

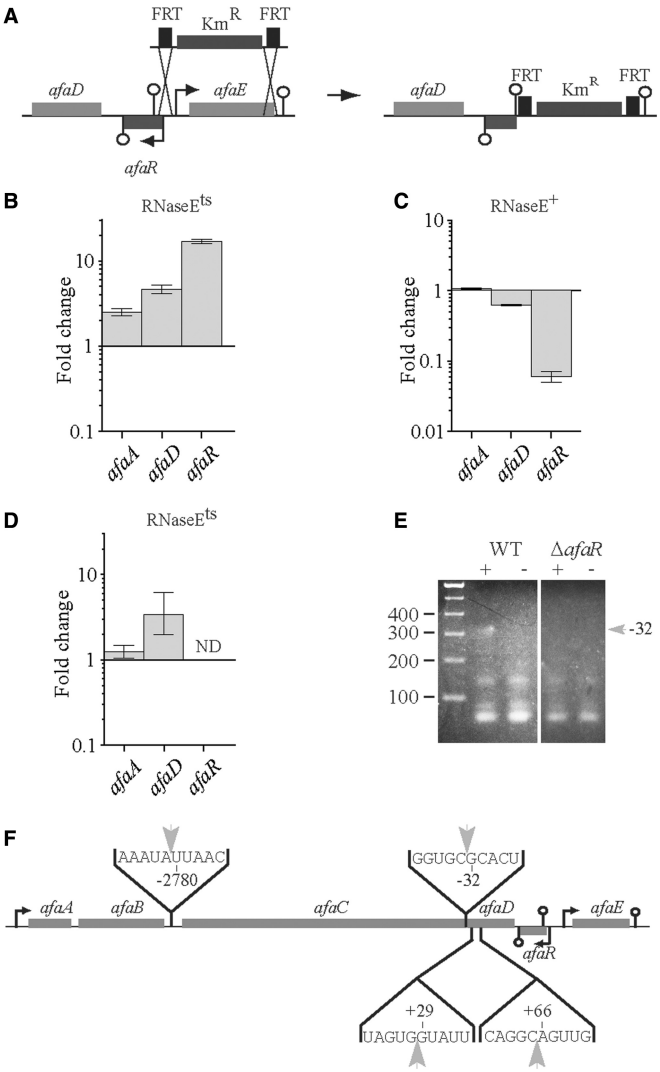

Figure 6. RNase E dependence of the regulation of *afaD* expression by AfaR. (**A**) Schematic representation of the construction of the pILL1323 plasmid by allelic exchange from pILL1320. qRT-PCR analysis confirmed an absence of *afaR* expression from pILL1323 (See Supplementary Figure S1). (**B–D**) Relative levels of the *afaA* and *afaD* mRNAs and AfaR sRNA, determined by qRT-PCR, in wild-type and RNase E^{ts} thermosensitive strains. The values for each gene are expressed as a ratio of expression at 42°C (low level of RNase E) to that at 30°C (high level of RNase E) for the N3431 + pILL1320 strain (B, [RNase E^{ts}]), the N3433 + pILL1320 strain (C, [RNase E^+]) and the N3431 + pILL1322 strain (D, [RNase E^{ts}]). (**E**) Circular RACE mapping of the *afaC* mRNA ends. Total RNA from *E. coli* BW25113 carrying pILL1320 (wild type) or pILL1323 (Δ*afaR*) was circularized by end-ligation with or without T4 RNA ligase (lanes + and −, respectively). The ligated 5′ and 3′ ends of the fragment were then amplified by RT-PCR. PCR products were analysed by electrophoresis in a 3% agarose gel. The band of interest was excised, cloned and sequenced. A ~310 bp DNA fragment (gray arrow) was more abundant after ligation treatment (lane +) in the wild-type strain than in the Δ*afaR* strain, indicating an AfaR-dependent amplification of the 5′ (cleavage at position -32) and 3′ ends of the *afaC* mRNA. This suggests that AfaR is involved in the RNase-dependent cleavage of the *afaABCD* mRNA. (**F**) Location of RNase cleavage sites in the *afaABCD* mRNA.

was more abundant at 37°C than 42°C (Figure 8), the apparent slightly lower levels of sRNA at 42°C are not significantly different from those at 37°C (without mRNA target), providing no evidence of lower stability. The stability of individual sRNAs varied widely, from highly stable to highly unstable, as reported for sRNAs in other bacteria (48,49). Taken together, these results indicate that AfaR transcription is controlled by temperature.

DISCUSSION

The invasion of a host by an ExPEC strain requires rapid adaptation to the new growth conditions associated with the transition from a free-living lifestyle to a host-associated state. This involves a reprogramming of the transcriptional activity of the bacterium. sRNAs function as gene regulators, relaying information from environmental sensors to response genes. We have previously identified ExPEC-specific sRNA genes, including four sRNA genes within a pathogenicity island of the *E. coli* AL862 strain, close to gene clusters encoding known virulence factors (6). Here, we characterized SQ109, a candidate sRNA renamed AfaR, which is encoded by a gene located in the *afa-8* gene cluster encoding a virulence-associated adhesive sheath. The σ^E- and temperature-dependent expression of AfaR is associated with the repression of AfaD-VIII invasin production, suggesting that AfaR is a stress-response regulator involved in the sensing of environmental stimuli relevant to host cell invasion.

The synthesis of outer membrane proteins (OMPs) is controlled by a plethora of sRNAs, acting as antisense RNAs, in gram-negative bacteria (50–52). Some sRNAs are associated with OMPs involved in virulence. The InvR, RnaG and VrrA sRNAs have recently emerged as key regulators of the OmpD, IcsA and OmpA OMPs in pathogenic *Shigella flexneri*, *S. typhimurium* and *V. cholerae*, respectively (21,13,53). We previously showed that the FimR sRNA controls the production of type 1 fimbriae (an adhesion factor of the chaperone/usher family) involved in the colonization and invasion of the urinary tract (54). This sRNA acts by an antisense mechanism, targeting the mRNA encoding the OMP FimD (6). In this study, we characterized AfaR, an sRNA that regulates *afaD* mRNA decay at the posttranscriptional level, thereby affecting AfaD-VIII invasin production. Recognition of the cellular β1-chain integrin by AfaD-VIII has been implicated in the internalization of beads coated with purified AfaD-VIII in cells derived from the uroepithelium, a target of *afa-8*-carrying strains, suggesting that such strains are invasive (25). The AfaD-VIII invasin is not strictly an OMP, but our data suggest that it is associated with the bacterial outer membrane. The identification of AfaR thus enlarges the sRNA family of antisense RNAs regulating cell surface proteins involved in pathogenicity.

During the biogenesis of adhesive structures of the chaperone/usher family at the cell surface, structural proteins are required in greater abundance than accessory proteins. Discoordinate gene expression is therefore essential. Regulatory mechanisms involving the RNase-dependent and -independent processing of mRNA from *E. coli* gene clusters encoding P-, S-fimbriae, CFA/I and F1845 adhesins have been reported (55–58). However, no sRNA has ever before been implicated in these processes. The identification of three sRNA gene candidates (*fimR*, *afaR* and *prsR*) overlapping adhesive structure-encoding gene clusters in a previous publication suggested a possible global involvement of sRNAs in the regulation of such virulence factors (6). In a putative sRNA-independent pathway of regulation, RNase E and RNase III have frequently been implicated in the processing of mRNAs for the biogenesis of adhesive structures (56,57). We therefore assessed the RNase dependence of the *afa-8* gene cluster (Supplementary Figure S3A) and found that RNases E, III and LS but not RNases G and P, played a potential role in mRNA processing. Our findings demonstrate that the cleavage activity of RNase E in processing the *afaABCD* mRNA upstream from *afaD* is dependent on AfaR. Other RNase-dependent cleavage events remain to be fully characterized and did not seem to involve the AfaR sRNA directly. This suggests that two separate sequential Afa-VIII biosynthesis regulation pathways may occur. The first involves cleavage of the *afaABCD* mRNA for regulation of the levels of each independent *afa* mRNA. In the second, the AfaR sRNA then specifically regulates *afaD* mRNA decay, probably switching AfaD-VIII invasin production on/off at the right time. A putative model of the regulation of the *afa-8* gene cluster is presented in Supplementary Figure S3B. Furthermore, the *afaD* mRNA was found to be subject to several AfaR-dependent RNase E processing events. These cleavages induced rapid *afaD* mRNA decay, but low levels of translation to generate AfaD protein nevertheless occurred. This suggests that AfaR is a finely tuned regulator that maintains AfaD protein synthesis in some conditions and facilitates invasion in certain circumstances. Thus, FimR and AfaR are the first sRNAs to be implicated in the regulation of OMPs exported by the chaperome/usher pathway.

Pathogenic bacteria are highly adaptive organisms in which a tight control of virulence gene expression is essential, and sRNAs are increasingly considered to be key elements of virulence control in *E. coli*. Our results indicate that production of the AfaD-VIII invasin is upregulated when AfaR is downregulated (Figure 8A and B), at temperatures above 37°C. The sRNA genes are, in general, regulated by various mechanisms unrelated to RNA stability, although sRNA regulation via transcript stability has not been ruled out in most cases (48,49). Temperature does not seem to affect AfaR stability or AfaR decay in the absence of its mRNA target, indicating that the stability of the AfaR transcript may be crucial for the regulation of *afaD* mRNA decay at any temperature. First, temperature changes may inform the bacterium that it is within the host, triggering production of the AfaD-VIII invasin. Second, it might indicate that *in vivo*, variation of the host temperature should not affect the AfaR regulatory pathway. Lastly, the bacteria can produce more AfaD-VIII invasin at higher

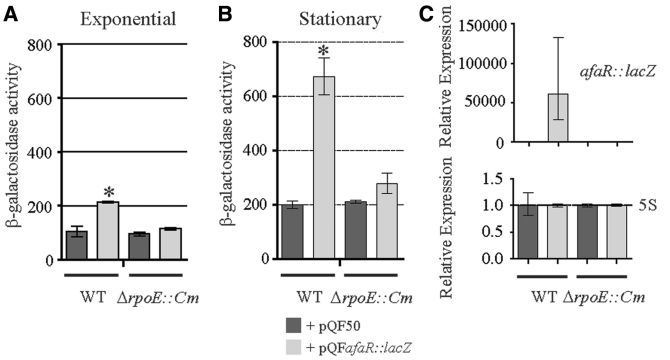

Figure 7. σ^E is required for induction of the transcription of *afaR*. (**A, B**) β-Galactosidase assay on *E. coli* MC1061 (WT) and CAG22216 (Δ*rpoE::Cm*) strains carrying the pQF50 or pQF*afaR::lacZ* plasmid. The promoter of the *afaR* gene was dependent on σ^E sigma factor in the (A) exponential and (B) stationary (24 h of culture) phases. The absence of σ^E totally abolished expression of the *afaR::lacZ* fusion gene. (**C**) Expression analysis of the *afaR::lacZ* fusion mRNA relative to the 5S gene in stationary phase. AfaR expression was abolished in the strain lacking σ^E. The asterisks indicate significant results.

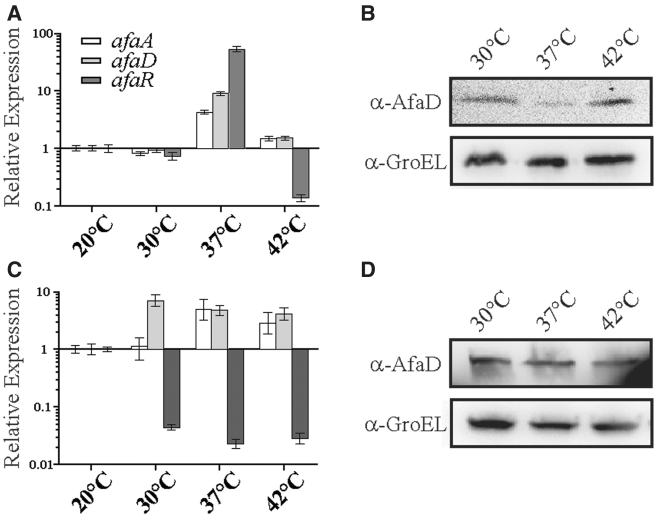

Figure 8. Control, by temperature, of the production of the AfaD invasin by AfaR regulation. (**A**) Assessment of the relative levels of expression of *afa-8* genes in strain BW25113 carrying pILL1320 grown to an OD_{600} of 0.6 at 30°C, 37°C and 42°C versus 20°C, as determined by qRT-PCR. The expression levels of all genes at 20°C were normalized to 1. (**B**) Western blot analysis of AfaD-VIII production by *E. coli* BW25115 carrying pILL1320 in the same growth conditions as (A). (**C, D**) as in (A, B), but with the BW25113 strain carrying pILL1323 (Δ*afaR::KmFRT*). GroEL was used as a loading control.

temperatures, presumably helping then to evade the host response to infection. Several putative temperature sensors might detect and relay information to the AfaR sRNA. We tested the hypothesis that the σ^E factor plays a crucial role in this process. Indeed, σ^E is a sigma factor associated with the extracytoplasmic/heat stress responses (59), as observed for expression of the P-fimbria gene cluster, which is controlled by temperature in a σ^E-dependent manner (60). More generally, the alternative σ^E factor upregulates the expression of many other sRNA genes, including MicA and RybB in *Salmonella* species (61) and, probably, FimR in ExPEC strains (6). Our data provide new evidence that the σ^E regulon may play a more important role in the virulence of gram-negative bacteria than previously suspected.

Expression of the *afa-8* gene cluster may also be controlled by the AfaF and AfaA proteins, which are homologues of the transcriptional regulatory proteins of the PapI/PapB family. We identified, *in silico*, six putative AfaA-binding sites (5′-CTATATTTT-3′ consensus sequence) in the *afaA* and *afaF* intergenic region and three other binding sites overlapping the promoter region of the *afaR* gene (Figure 4A). These findings strongly suggest that *afaR* transcription may also be dependent on these regulatory proteins. We also found that AfaR expression was strongly downregulated at 42°C, suggesting that AfaR transcription may be dependent on another, as yet undetermined mechanism. Data from several studies have shown the CpxAR two-component systems control the production of curli and P-fimbriae in uropathogenic *E. coli*, of type IV bundle-forming pili in enteropathogenic *E. coli*, and downregulate the expression of σ^E-dependent genes, rendering them of interest for future studies (60).

Comparative genomics of the *afaD*/*afaE* intercistronic region of the *afa-8* gene cluster showed it to be very specific in size and composition, and no sequence related to the *afaR* gene was detected in the other gene clusters of the *afa* family. Thus, AfaR seems to be specific to the *afa-8* gene cluster. We also found that the 3′ end of the *afaR* gene displayed sequence identity to an IS2 insertion sequence, at both the DNA and protein levels. There is therefore evidence to suggest that *afa-8* evolved from its ancestor at least partly through IS2-mediated disruption of the *afaD*/*afaE* intercistronic region. Pseudogenization of the IS2 transposase gene may then have led to the creation of the *afaR* gene. This reorganization of the intercistronic sequence also seems to have had consequences for the transcription units specific to the *afa-8* gene cluster. Indeed, only in *afa-8* was it was possible to identify an *afaE* promoter and a rho-independent terminator for the *afaABCD* mRNA. These promoter and terminator sequences may have been acquired from the IS2 sequence, as previously reported following the insertion of IS2 into the *ampC* promoter (62) or of IS3 insertion into the 5′ end of *argE* (63). IS elements have been shown to play a role in the dispersal of SprA sRNA genes between strains of the genus *Staphylococcus* (9). Finally, our study suggests that some of the sRNA genes in *E. coli* may be derived from IS elements.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2, Supplementary Figures 1–3, Supplementary Results and Supplementary References [64–66].

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