Hfq-dependent mRNA unfolding promotes sRNA-based inhibition of translation

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Abstract

Small RNAs post-transcriptionally regulate many processes in bacteria. Base-pairing of sRNAs near ribosome-binding sites in mRNAs inhibits translation, often requiring the RNA chaperone Hfq. In the canonical model, Hfq simultaneously binds sRNAs and mRNA targets to accelerate pairing. Here, we show that the Escherichia coli sRNAs Omra and Omrb inhibit translation of the diguanylate cyclase DgcM (previously: YdaM), a player in biofilm regulation. In Omra/B repression of dgcM, Hfq is not required as an RNA interaction platform, but rather unfolds an inhibitory RNA structure that impedes Omra/B binding. This restructuring involves distal face binding of Hfq and is supported by RNA structure mapping. A corresponding mutant protein cannot support inhibition in vitro and in vivo; proximal and rim mutations have negligible effects. Strikingly, Omra/B-dependent translational inhibition in vitro is restored, in complete absence of Hfq, by a deoxyoligoribonucleotide that base-pairs to the biochemically mapped Hfq site in dgcM mRNA. We suggest that Hfq-dependent RNA structure remodeling can promote sRNA access, which represents a mechanism distinct from an interaction platform model.

Keywords biofilm; Hfq; post-transcriptional regulation; RNA structure; sRNA

Introduction

Bacterial antisense RNAs were initially found and described as regulators in accessory elements: plasmids, phages, and transposons (Wagner et al., 2002; Wagner & Romby, 2015). These cis-encoded antisense RNAs are encoded overlapping—in opposite orientation—their single target gene and thus have complete complementarity (Waters & Storz, 2009; Wagner & Romby, 2015; and references therein). Subsequently, numerous genomically encoded so-called small RNAs (sRNAs) were discovered and are part of a large repertoire of post-transcriptional regulators in all bacteria and archaea (Beisel & Storz, 2010; Gottesman & Storz, 2011; Barquist & Vogel, 2015; Wagner & Romby, 2015; Holmqvist & Wagner, 2017). This heterogeneous class of RNAs impacts on global regulatory circuits in metabolism, virulence, stress responses, and motility/biofilm formation decisions. A striking feature involves targeting of multiple genes within the same network. This relies on properties of trans-encoded sRNAs: Since many sRNA genes do not overlap with target genes, base-pairing is non-contiguous and limited. Bacterial sRNAs can promote translational activation or, more frequently, repression (Papenfort & Vanderpool, 2015; Wagner & Romby, 2015; Carrier et al., 2018). Furthermore, target mRNAs can become stabilized or undergo facilitated decay (Lalaouna et al., 2013; Papenfort et al., 2013).

In many but not all bacteria, trans-encoded sRNAs require the RNA chaperone Hfq for efficient regulation (Vogel & Luisi, 2011; Holmqvist & Vogel, 2018; Woodson et al., 2018). Hfq is a ring-like homohexamer with at least three distinct RNA-binding surfaces (Updegrove et al., 2016; Santiago-Frangos & Woodson, 2018). The distal face of Escherichia coli Hfq recognizes A-rich sequences, or (AAN)n motifs, and primarily binds mRNAs (Link et al., 2009; Robinson et al., 2014). In contrast, most sRNAs bind the proximal face via the poly-U tails of their Rho-independent terminators (Otaka et al., 2011; Ishikawa et al., 2012), with specific recognition of the 3’ hydroxyl group (Sauer & Weichenrieder, 2011). Additional contacts are provided between internal U/A sequences and arginine patches on the lateral binding surface/rim (Sauer et al., 2012). Enterobacterial Hfq also carries intrinsically disordered C-terminal domains (CTD; Beich- Frandsen et al., 2011) whose function has been controversial. CTDs may aid recruitment of sRNAs, specifically RydC, to Hfq (Dimastrogiovanni et al., 2014). A recent study suggests their importance for the rapid release of bound RNAs (Santiago-Frangos et al., 2016). Overall, Hfq is regarded as a platform on which RNAs meet for interaction. For this to happen on a biologically relevant timescale, RNAs must rapidly exchange on the limiting Hfq pool. RNA concentration-driven cycling on Hfq has strong support from in vitro and in vivo experiments (Fender et al., 2010; Salim & Feig, 2010; Hussein & Lim, 2011; Moon & Gottesman, 2011; Olejniczak, 2011; Wagner, 2013).

Interaction with Hfq protects many RNAs from rapid degradation (Moll et al., 2003a; Holmqvist et al., 2010) and promotes the annealing of sRNAs and their mRNA targets (Mellor et al., 2002; Zhang et al., 2002; Lease & Woodson, 2004; Fender et al., 2010; Wroblewska & Olejniczak, 2016). Several non-exclusive models...
suggest how Hfq can facilitate intermolecular base-pairing. Simultaneous binding of two RNA molecules to an Hfq hexamer could help their interaction through “molecular crowding” (Rajkowitsch & Schroeder, 2007). Hfq binding may also entail structural rearrangements in RNAs, to unfold an otherwise inaccessible seed sequence in an sRNA (Zhang et al., 2002; Gorski et al., 2017) or possibly a sequenced target sequence in an mRNA. The latter has previously been proposed for sodB mRNA (Geissmann & Touati, 2004), but, as will be detailed in the Discussion, lacks unambiguous experimental support and has been challenged (Hao et al., 2011). Another mechanism specifically moves the seed region of an sRNA into close proximity to its complement in the mRNA (Vogel & Luisi, 2011; Gorski et al., 2017). For example, the rpoS mRNA folds around Hfq, through contacts with distal and rim surfaces, into a compact ternary structure that positions the base-pairing region adjacent to the complementary site within the sRNA DsrA (Peng et al., 2014a,b). Interaction with the Hfq rim can also position sRNAs for optimal base-pairing, as shown for RydC-cfa (Dimastrogiovanni et al., 2014). Besides positioning RNAs for interaction, the conserved arginine patches on the Hfq rim apparently play a role in duplex formation by overcoming electrostatic barriers to helix nucleation (Panja et al., 2013, 2015).

We and others have been interested in two sequence-similar sRNAs, OmrA and OmrB, which act on multiple target mRNAs to regulate outer membrane and surface protein expression (Guillier & Gottesman, 2006, 2008; Holmqvist et al., 2010; Mika & Hengge, 2014; Brosse et al., 2016; Jagodnik et al., 2017). Their genes (Argaman et al., 2001; Wassarman et al., 2001; Vogel et al., 2003) are transcriptionally activated by the OmpR-EnvZ two-component system, e.g., under high osmolarity conditions (Guillier & Gottesman, 2006; Brosse et al., 2016). OmrA, but not OmrB, is additionally under the control of the stress sigma factor σE (rpoS; Lévi-Meyrueis et al., 2014; Peano et al., 2015; Colgan et al., 2016). Both sRNAs associate with Hfq in vitro (Holmqvist et al., 2010) and in vivo (Wassarman et al., 2001; Zhang et al., 2003; Chao et al., 2012; Tree et al., 2014; Holmqvist et al., 2016); the sRNAs are destabilized in Δhfq cells (Holmqvist et al., 2010). OmrA and OmrB are redundant on most targets, using their almost identical 5′ tails for base-pairing. Some validated target mRNAs encode the outer membrane proteins CirA and OmpT, their own activator OmpR (Guillier & Gottesman, 2008), the flagellar regulator FlhD (De Lay & Gottesman, 2012), and CsgD (Holmqvist et al., 2010), the master transcriptional activator of the genes for the biofilm components curli and cellulose (Hammar et al., 1995; Römling, 2005). The regulation of csgD by OmrA and OmrB (Holmqvist et al., 2010) and by four subsequently reported sRNAs (Jorgensen et al., 2012; Mika et al., 2012; Thomason et al., 2012; Bordeau & Felden, 2014) has highlighted the importance of tight regulation of biofilm formation. In addition to post-transcriptional control by sRNAs, csgD expression is extensively regulated at the level of transcription, e.g., by the OmrAB-regulated transcription factor OmpR, and DgcM (previously: YdaM; Hengge et al., 2015; Fig 1A). DgcM, a c-di-GMP-producing diguanylate cyclase (DGC), is part of a c-di-GMP-dependent switching module involved in the signaling cascade that controls curli synthesis. The phosphodiesterase (PDE) DpeR (a.k.a. YciR) directly induces DgcM until the levels of the second messenger c-di-GMP are sufficient for it to function as a PDE. DgcM then produces more c-di-GMP and, through a double-negative feedback loop, activates MlrA (Lindenberg et al., 2013). DgcM, MlrA, and σE are all required for transcription of csgD. CsgD in turn activates transcription of the csgBAC operon that encodes curli proteins (Hammar et al., 1995; Fig 1A).

In this study, we identified dgcM as an additional OmrA/B target, upstream in the curli/biofilm pathway. Unexpectedly, our results

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**Figure 1. A new OmrA and OmrB target, in addition to csgD and ompR, that affects curli synthesis.**

A Schematic overview of part of the biofilm regulatory network, highlighting the roles of OmrA and OmrB. Gray arrows: transcriptional activation, black lines with bars: post-transcriptional negative regulation, dotted lines: direct protein–protein or protein–c-di-GMP interactions.

B Congo red plates with colonies of Escherichia coli strain MC4100 relAΔ and its target mutation csgDΔ-ompRΔmut2 derivative, harboring sRNA-producing plasmids pOmrA, pOmrB, or an empty vector (pControl). Incubation at 28°C for 48 h.

C Schematic showing published interaction sites between OmrA or OmrB and targets csgD or ompR (Guillier & Gottesman, 2008; Holmqvist et al., 2010). Mutations introduced to disrupt base-pairing are in gray boxes.
suggest a new mechanism by which Hfq promotes regulation. Here, rather than acting canonically as a platform for simultaneous interactions that facilitate sRNA-mRNA pairing, Hfq is primarily needed to render the mRNA target accessible for sRNA binding.

Results

OmrA and OmrB have multiple targets in the same regulatory pathway

We previously showed that OmrA/B inhibit the synthesis of CsgD (Holmqvist et al., 2010). OmrA/B also repress the synthesis of OmpR (Guillier & Gottesman, 2008), a transcriptional activator of csgD (Römling et al., 1998; Vidal et al., 1998; Prigent-Combaret et al., 2001; Gerstel et al., 2003; Ogasawara et al., 2010; Fig 1A). As a result of the dual repression of these biofilm activators, ectopic overexpression of the sRNAs strongly decreased fluorescence compared to an empty vector control (Fig 2B), indicating direct repression of DgcM synthesis by the sRNAs. When the same plasmids were introduced into a Δhfq background, regulation was lost (Fig 2B). Note, however, that, as for csgD (Holmqvist et al., 2010), uninhibited dgcM expression was ≈3-fold lower in a Δhfq strain than an hfq+ background (Appendix Fig S2A). Thus, as in many trans-encoded sRNA-target interactions, Hfq is required for regulation. To assess whether inhibition of dgcM is dependent on the predicted base-pairing interaction, Western blot analysis was performed on cell lysates from E. coli cells expressing dgcM with a C-terminal 3xFLAG sequence, combined with OmrA or OmrB overexpression vectors, or their mutant variants OmrA_M2, OmrB_M2 (Fig 2A). Overexpression of wild-type (wt) OmrA or OmrB strongly decreased DgcM-3xFLAG signals, congruent with the GFP fusion results (Fig 2B and C). As expected, mismatched OmrA_M2 or OmrB_M2 did not affect DgcM expression, supporting the importance of these nucleotides in base-pairing to the dgcM mRNA (Fig 2A and C). A compensatory mutation in dgcM, designed to base-pair with OmrA_M2 and OmrB_M2, restored regulation for OmrB_M2, but not OmrA_M2 (Fig 2A and C). Mobility shift assays showing binding of OmrA_M2 to both the wt dgcM and dgcM_M2 mRNAs (Appendix Fig S3). We therefore hypothesize that OmrA_M2 preferably binds to a second, likely regularly inert site. Interestingly, when we supplemented a strain carrying both csgD and ompR target mutations (Fig 1C) with a plasmid encoding dgcM_M2, thus rendering all known OmrA/B biofilm targets unregulated, biofilm formation was restored in the presence of OmrA, but not OmrB (Appendix Fig S4A). It is therefore possible

![Figure 2. OmrA and OmrB inhibit dgcM expression in vivo by direct base-pairing in an hfq-dependent manner.](image-url)

A Predicted base-pairing between dgcM mRNA and OmrA/OmrB. Gray boxes show the M2 point mutation introduced to disrupt the interaction. The UTR is 74 nt long (to AUG), and the interaction site is numbered.

B Relative fluorescence/OD400 of cells of Escherichia coli strain MC4100 rel-ΔΔomrAB or MC4100 rel-ΔΔomrAB Δhfq harboring the translational fusion plasmid pDgcM::gfp in combination with plasmids expressing OmrA, OmrB, or an empty control vector, after 16 h of growth at 37°C. Error bars SD, N = 3. Average fluorescence intensities (a.u./OD400) used to calculate ratios can be found in Appendix Fig S2A. Note that absolute dgcM::gfp expression values are ≈3-4-fold lower in the Δhfq strain (Appendix Fig S2B).

C Western blot analysis of cell lysates from strains MC4100 rel-ΔΔomrAB harboring plasmids pOmrA, pOmrB, or mutant variants as indicated, in combination with a plasmid expressing C-terminal FLAG-tagged dgcM or dgcM_M2. As a loading control, part of a protein-stained gel is shown below.
that OmrB may target additional biofilm-related mRNAs. Taken together, our results indicate that OmrA and OmrB repress DgcM output by direct base-pairing interaction within the early coding region of the mRNA and that Hfq is required for their effect.

**OmrA and OmrB inhibit dgcM translation in vitro**

The OmrA/OmrB interaction site on dgcM is located immediately downstream of the "5-codon window" within which sRNAs are believed to interfere with 30S ribosomal subunit binding (Bouvier *et al*., 2008). Thus, we tested whether OmrA and OmrB can block initiation complex formation on dgcM mRNA. As expected, a characteristic 30S/5sRNA Hfq-dependent reverse transcription stop ("toeprint") at position +15 was observed, which was absent upon inclusion of OmrA or OmrB (Fig 3A); an unrelated sRNA, IstR-1 (Vogel *et al*., 2004), failed to inhibit the dgcM toeprint.

Translational level regulation was further tested with in vitro assays. Here, dgcM::FLAG and ompA::FLAG mRNAs were used as templates, the latter serving as internal control; this mRNA is not subject to OmrA/B regulation. Upon adding OmrA or OmrB to the reaction, a slight reduction of DgcM translation was observed, with OmrA being reproducibly more effective than OmrB (Fig 3B). Addition of Hfq greatly enhanced the inhibitory effect of both sRNAs, so that DgcM was essentially absent (Fig 3B). In *vivo*, Hfq is required for the stability of OmrA and OmrB (Holmqvist *et al*., 2010). However, since RNAs are entirely stable in the PURExpress® In Vitro Protein Synthesis Kit (Sharma *et al*., 2007), this striking regulatory dependence implies a key role for Hfq in dgcM mRNA-OmrA/B duplex formation.

**OmrA/B and dgcM mRNA interaction sites**

The above results suggest that dgcM mRNA and OmrA/B directly base-pair with each other, most likely in close proximity of, or overlapping, the ribosome-binding site (RBS). To precisely map this interaction, a footprinting analysis was conducted by enzymatic and chemical probing. 5’-end-labeled dgcM mRNA, ±Hfq, and/or OmrA/B was subjected to limited digestion (on average < 1 cut/RNA molecule) with RNase T1 (specific for unpaired G residues) or lead(II) acetate (specific for single-stranded nucleotides; Fig 4A). Binding of Hfq alone protected residues 48–64 (lead-II cleavage) as well as G54 (RNase T1; Fig 4A and B). This region of the dgcM mRNA features the typical (AAN)n motifs known to bind the distal face of Hfq hexamers (Link *et al*., 2009; Robinson *et al*., 2014). Hfq binding also induced minor long-range structural rearrangements, e.g., 5′ of the main Hfq-binding site (G32–G39; Fig 4A). Importantly, Hfq binding entailed an opening of the small hairpin that contains the RBS of the dgcM mRNA, indicated by enhanced T1 cuts of G66–68 within the SD sequence, and at G83 on the opposite side of the stem. We also observed that residues G101 and G102 were protected from RNase T1 (Fig 4A, lane 6). Unexpectedly, structural probing done with Hfq and either OmrA or OmrB revealed the presence of two adjacent sRNA-binding sites (Fig 4A, lanes 9–10, and 15–16, indicated by red and blue boxes). Protection of site 1 was identical for both OmrA and OmrB and concurred with the computationally predicted binding site. It spans from position U91 to G102, as shown by complete protection of G101 and G102 from T1 cleavage, and lead-II protection from U91 to G102 (Fig 4A and B). Site 2 differs between the sRNAs. Both OmrA and OmrB base-pair from U121 to G125, since G124, G125 (T1), and U121 to G125 (lead-II) were fully protected in the presence of Hfq and the sRNAs (Fig 4A, lanes 9–10). Binding of OmrA, but not OmrB, extended protection to residues C110 to U123 (lead-II) and G115 (T1). Incidentally, both OmrA/B binding sites are supported by RIL-seq results (Holmqvist *et al*, 2010). Corresponding and complementary probing was done with labeled OmrA and OmrB (Appendix Fig S5). As expected, both sRNAs bind through their conserved 5′ tails, as seen on other mRNA targets (Holmqvist *et al*., 2010).

To confirm OmrA/B binding to sites 1 and 2, we performed mobility shift assays with a shorter end-labeled dgcM mRNA and OmrA or OmrB. DNA-oligos complementary to site 1 or site 2 (Appendix Fig S6 and Table S1) were pre-annealed to the mRNA to...
block the accessibility of the respective sites to base-pairing of the sRNAs. When site 1 was blocked, OmrA—but not OmrB—could still bind site 2 (Fig 5). Blocking site 2 still permitted both sRNAs to bind site 1. When both sites were blocked, neither of the sRNAs could bind. Interestingly, in the absence of oligos, OmrA was more efficient in the gelshift than OmrB. Furthermore, a super-shift was observed with OmrA-dgcM mRNA, indicating that two OmrA molecules were bound to one dgcM mRNA. Though binding between OmrA and site
2 clearly occurs in vitro (Figs 4 and 5), this interaction is likely not of regulatory importance, since point mutations introduced in site 1 alone eliminated regulation completely (Fig 2A and C).

**Hfq-dependent restructuring of the dgcM mRNA is essential for regulation by OmrA and OmrB**

Inspection of the predicted dgcM 5’ structure based on footprinting results (Fig 4) and the mFold algorithm (Zuker, 2003) suggested that the presumed seed region within the OmrA/B binding site 1 is partially sequestered in a hairpin (Fig 4B, upper structure), likely rendering it difficult to access by the sRNAs. Based on footprinting results (Fig 4A), we hypothesized that Hfq binding unfolds this structure to facilitate sRNA-mRNA duplex formation (Fig 4B, lower structure). If so, this might explain the strong dependence on Hfq for regulation. However, Hfq is also known to enhance base-pairing rates by bringing sRNA and mRNA target into close proximity, since it can bind two RNAs simultaneously through its different interaction surfaces. To tease apart the contributions of Hfq in these non-exclusive models, we performed translation assays, adding Hfq mutant proteins with specific impairments in the proximal (St_Hfq102_F42A), distal (St_Hfq102_Y25A_Q52A), or rim (St_Hfq102R16S_R17A_R19A_K47E) interaction surface (Sauer et al, 2012); the Hfq mutant proteins are subsequently referred to as Hfq-dist, Hfq-prox, and Hfq rim. As in Fig 3B, ompA mRNA was used as an OmrA/B-independent internal control.

Figure 6A shows that, at the concentrations used, OmrA or OmrB alone do not significantly inhibit DgcM translation, whereas the inclusion of wt Hfq drives DgcM output down to barely detectable levels. The dgcM mRNA, carrying an A-rich Hfq-binding motif (+45–65, Fig 4B), is expected to interact with the Hfq distal face (Link et al, 2009). Assuming that Hfq-mediated opening of the inhibitory structure in dgcM mRNA is important for OmrA/B binding, mutations affecting the distal face should impair regulation. Indeed, supplementing the *in vitro* translation reaction with a distal face mutant protein strongly impeded OmrA-dependent regulation, nearly mirroring the absence of Hfq (Fig 6A). In agreement, mobility shift assays showed that, in the presence of Hfq-dist, very little dgcM-OmrA complex was formed (Appendix Fig S7). By contrast, Hfq-prox or Hfq rim supported strong inhibition by OmrA, and less so by OmrB. Hence, these surfaces are not strictly needed for Hfq’s regulation-enhancing effect, whereas distal face interaction on dgcM mRNA is required for efficient regulation.

The results in Fig 6A alone do not exclude a simultaneous interaction of either sRNA with Hfq as important for regulation. However, since the proximal face and rim are preferred interaction surfaces for most sRNAs, including OmrA and OmrB (Schu et al, 2015), Hfq-prox and Hfq rim should not interact with the sRNAs, but be still capable of opening the inhibitory structure in dgcM mRNA. Mobility shift assays confirmed that OmrA does not bind to Hfq rim but forms a complex with Hfq-prox (Appendix Fig S7). This indicates that for OmrA, akin to some sRNAs like RyhB and RprA, the rim suffices for stable interaction (Sauer et al, 2012). More importantly, Hfq rim, unable to bind OmrA (Appendix Fig S7), nevertheless supports strong OmrA-dependent inhibition of DgcM translation (Fig 6A), suggesting that Hfq rim acts here through its intact distal face, similar to Hfq-wt. Mobility shift assays indeed show Hfq rim promoted OmrA-dgcM mRNA complex formation, but—unlike with Hfq-wt and Hfq-prox—no slowly migrating ternary complexes (Appendix Fig S7). Taken together, Hfq distal face binding to dgcM mRNA is most important for regulation, whereas binding of Hfq to OmrA/B, and its effect on annealing, may be less important or not required.

**Forced opening of the inhibitory structure is sufficient to enhance regulation**

Based on the above results, a forced opening of the structure that hinders OmrA/B access might bypass the Hfq requirement. We chose to mimic Hfq binding by annealing of a DNA-oligo with complementarity to the Hfq-binding site in dgcM (Appendix Table S1 and Fig S6). Indeed, a dgcM mRNA annealed to the Hfq-mimic oligo was substantially inhibited by both OmrA and OmrB in the complete absence of Hfq (Fig 6B). The inhibition by OmrA/B was roughly comparable to that obtained in the presence of Hfq (cf. Fig 6B and A, lanes 4–6) and significantly stronger than in its absence (Fig 6A, lanes 1–3). Altogether, this suggests that—in the case of OmrA/B regulation of dgcM—an “interaction platform” involving the binding of both interacting RNAs is not required. Rather, the major role of Hfq lies in converting
an mRNA structure element into an sRNA-accessible site (cf. structures in Fig 4B). This inference predicts that OmrA/B association rates should be higher upon structure opening. Mobility shift assays with labeled OmrA and dgcM mRNA, or dgcM mRNA with the pre-annealed mimic oligo, indeed showed association rates effects; second-order binding rate constants for OmrA to dgcM were about three times higher in presence of the oligo (Appendix Fig S8).

In conclusion, the Hfq requirement for regulation can be largely bypassed by binding of a DNA-oligo that opens a local structure in the dgcM mRNA. Thus, the predominant function of this RNA chaperone lies in unfolding dgcM mRNA to render its target region more rapidly accessible to OmrA/B.

**The Hfq distal surface is required for OmrA/B-dependent regulation of DgcM in vivo**

To assess the contribution of the Hfq-binding surfaces on regulation in vivo, we introduced the dgcM::gfp translational fusion plasmid into E. coli strains harboring chromosomal hfqF42A, hfq-Y25D, hfq-R16S_R17A_R19A_K47E mutant alleles, as well as in a wild-type and a Δhfq strain. Flow cytometric analyses of these cells, carrying or lacking omrA or omrB expression plasmids, showed that—in line with Fig 2—regulation was abolished in a Δhfq background. Interestingly, regulation was also lost in the distal face mutant (hfq-Y25D) strain (Fig 7A), in spite of even slightly higher sRNAs levels than in the wild-type hfq strain (Fig 7B; cf. hfq wt and hfq-dist). These results are congruent with the in vitro experiment in Fig 6A and support that the Hfq distal face is required for remodeling of the dgcM mRNA and regulation also in an intracellular environment. In apparent contrast to the in vitro translation experiment (Fig 6A), hfq proximal and rim mutations fail to support regulation of dgcM by OmrA and OmrB in vivo (Fig 7A). This is however tentatively explained by the strongly reduced stability of the sRNAs in these mutant strains (Fig 7B).

In conclusion, the distal face of the Hfq hexamer is essential for OmrA/B-dependent regulation of dgcM both in vitro and in vivo. This likely reflects that the Hfq distal surface interaction with the dgcM mRNA promotes local unfolding, exposing the seed region for sRNA-mRNA duplex formation.

**Discussion**

In this report, we identified dgcM, encoding an upstream regulator of the biofilm signaling pathway in E. coli, as a target of the sRNAs OmrA and OmrB, thus adding to the complexity of the sRNA network in control of biofilm formation (Boehm et al, 2010; Fig 1A). As suggested by Mika and Hengge (2013, 2014), OmrA/B regulation of dgcM, as well as csgD, might be important to prevent expression of biofilm components under stationary-phase conditions where RpoS is active, and may be critical in the decision to convert from a motile planktonic to a biofilm lifestyle. Inhibition of DgcM translation in vivo and in vitro occurs by direct base-pairing within the early coding region, as defined by effects of mutations in site 1 (Fig 2). Though OmrA/B pulse expression causes a strong decrease in dgcM mRNA levels (Hoekzema, Holmqvist, in preparation), the demonstration that the sRNAs inhibit initiation complex formation (Fig 3A) and in vitro translation (Fig 3B) indicates translation inhibition to be the dominant effect. As is true for many other sRNA-mRNA combinations, regulation of dgcM by OmrA/B is dependent on the RNA chaperone Hfq. This applies not only in vivo, where Hfq is needed to stabilize these sRNAs (Holmqvist et al, 2010), but also in vitro (Fig 6A) where stabilization effects are ruled out.

Enzymatic and chemical structure probing of the dgcM mRNA combined with in silico structure predictions (mFold; Zuker, 2003) indicated that OmrA/B binding site 1, on which regulation depends.
mimic oligo, which base-paired to the mapped Hfq-binding site in remodeling. Strikingly, when this protein was substituted by an Hfq-sRNA access needs mRNA remodeling by Hfq 
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The capability of the RNA chaperone Hfq to locally unwind and/or restructure bound RNAs is well established and hardly surprising (e.g., Woodson et al, 2018). Remodeling has been demonstrated for the sRNAs OxyS, RprA, and DsrA (Zhang et al, 2002; Vecerek et al, 2008; Ribeiro Ede et al, 2012; Henderson et al, 2013), as well as the ompA, sodB, and mutS mRNAs (Moll et al, 2003b; Geissmann & Touati, 2004; Chen & Gottesman, 2017). In the case of mutS, Hfq inhibits translation through both sRNA-dependent and sRNA-independent mechanisms. While the sRNA ArcZ inhibits translation by base-pairing to the mutS 5’UTR, Hfq distal face binding remodels the mRNA leader to induce an inhibitory structure in the translation initiation region (Chen & Gottesman, 2017). However, these structural changes do not seem to affect inhibition of mutS by its regulator ArcZ; downregulation by ArcZ is almost as strong in an Hfq distal face mutant strain as in the wild-type (Chen & Gottesman, 2017).

Hfq-dependent remodeling of mRNA as a requirement for sRNA interaction has specifically been proposed for RyhB regulation of sodB (Geissmann & Touati, 2004). We noted however that, while Hfq-dependent structure remodeling of the sodB mRNA was demonstrated, the expected faster binding of RyhB to sodB mRNA in the presence of Hfq has not been experimentally tested. Neither has the effect of Hfq on translation initiation, or overall translation, been examined, thus rendering the mechanistic interpretations ambiguous (Geissmann & Touati, 2004). Moreover, the conclusions from this publication are at odds with another study of the same sRNA-target system (Hao et al, 2011). Here, a truncated RyhB variant, RyhBt, which is entirely Hfq-independent and stable in the absence of Hfq, was fully active in sodB expression control in vivo. Thus, whether remodeling of sodB mRNA is the critical event for regulation by RyhB, as proposed in Geissmann and Touati (2004), needs to be addressed by further experiments. Hfq-induced structural changes have also been reported for ompA mRNA, but it remains unknown whether they are required for duplex formation with targeting sRNAs (Moll et al, 2003b).
The cis-encoded RNA-IN and RNA-OUT system (transposon Tn10/I510) also involves secondary structure rearrangements brought about by Hfq binding, suggested to explain how Hfq facilitates RNA-IN/OUT pairing (Ross et al., 2013). So far, the functional implications of these rearrangements are not entirely clear, in particular since an Hfq-independent binding pathway involving kissing loop interactions is known (Ross et al., 2013), and protein-free association rate constants of RNA-IN and RNA-OUT (0.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}; Ma & Simons, 1990) are > 100-fold faster than those typical for trans-encoded sRNAs (Wagner & Romby, 2015). This is different from our model, in that functional OmrA/B-dgcM mRNA interactions barely occur in the absence of Hfq (Appendix Fig S7). Though we propose that dgcM-OmrA/B is a first case in which this mechanism has been experimentally validated, other systems, like those above and others, may indeed work similarly. For example, regulation of et al.

by RprA (Mika et al., 2012) appears to occur at a binding site close to that of OmrA/B, and hence might conform to the same model.

A surprising finding was the ability of OmrA/B to protect dgcM mRNA from in vitro cleavage at two distinct but adjacent sites (Fig 4). Two-site recognition by one sRNA has previously been reported for the lpxR and nhaB mRNAs. In both cases, binding to one site overlapping the RBS blocks translation by competing with initiating ribosomes, whereas binding to the second site far downstream in the coding region induces RNAse E-mediated cleavage (Corcoran et al., 2012; Chao & Vogel, 2016). Here, only OmrA/B binding site 1 (nt 88–102) in dgcM is associated with inhibition (Fig 3A and B) since mutations that weaken this interaction suffice to abolish regulation in vivo (Fig 2A and C). In light of these data, a regulatory role for site 2 seems unlikely.

In conclusion, the present analysis identified OmrA/B as regulators of yet another target within the biofilm pathway of E. coli, DgcM. More importantly, we uncovered a different and new mechanism of action for Hfq. Instead of acting as a canonical platform for simultaneous sRNA-mRNA binding, Hfq acts here—exclusively or at least primarily—by opening a local target structure in the dgcM mRNA to provide access to the regulatory sRNAs. The in vitro data strongly support these conclusions, although it cannot be ruled out that, e.g., a platform role of Hfq may contribute to in vivo activity. We suggest that it might be fruitful to search for similar examples of Hfq-dependent regulation in which this protein’s RNA structure-remodeling activity, rather than its annealing function or duplex-stabilizing effect, plays the dominant role.

Materials and Methods

Chemicals, reagents, and oligodeoxyribonucleotides

Growth media components were purchased from Oxoid. Oligodeoxyribonucleotides/primers (Appendix Table S1) and chemicals were purchased from Sigma-Aldrich, and reagents from Thermo Fisher Scientific.

Growth conditions

Bacterial cells were grown aerobically at 37°C in L Broth. When applicable, antibiotics were added at 100 (ampicillin), 50 (kanamycin), or 15 μg/ml (chloramphenicol and tetracycline). For detection of curli expression, Congo Red plates (LA plates without NaCl, 20 μg/ml Congo red, 10 μg/ml Coomassie Brilliant Blue G) were used. Plates were incubated at 28°C for 24 h.

Bacterial strains and plasmids

All strains used in this study are derivatives of E. coli K12 MC4100 relA+, unless otherwise stated. Strains and plasmids are listed in Appendix Tables S2 and S3, respectively.

Plasmids pOmrA, pOmrB, and pControl were previously published (Holmqvist et al., 2010). Plasmid pDgcM-GFP was constructed by inserting a NsiI/Nhel-digested PCR product (primers EHO-759/EHO-760) into NsiI/Nhel-digested pXG10-SF, fusing 641 bp from the transcription start site to gfp. pXG10-SF is identical to pXG-10 (Urban & Vogel, 2007) except for substitution of gfp by sGFP. Plasmid pDgcM::3xFLAG was created by insertion of dgcM::3xFLAG between the lac promoter sequence and the terminator sequence on the pXG-10 vector, thereby replacing gfp: the dgcM::3xFLAG fragment was amplified from pET52-b_dgcM_FLAG (see below) with primers MHO-259 and MHO-260, introducing XbaI and PstI restriction sites, and after digestion with both enzymes, ligated into XbaI/Nhel digested pXG-10. Plasmids pOmrA_M2 and pOmrB_M2 were created using inverse PCR mutagenesis, with the desired mutations included in the primers (MHO-221/MHO-223 for OmaR/OmrB, respectively, and MHO-222). Briefly, after PCR amplification with the phosphorylated primers, pOmrA or pOmrB template DNA was digested by DpnI. PCR product was purified (GeneJET PCR Purification Kit Thermo Fisher Scientific) and religated with Ready-to-go T4 ligase (Amersham). The pDgcM_M2::3xFLAG plasmid was created with primers MHO-217 and MHO-218 on pDgcM::3xFLAG as template. To create a dgcM strain, dgcM was replaced by a FRT-flanked Amp R cassette by Lambda Red recombination (Yu et al., 2000) using primers MHO-094 and MHO-095. For dgcM transcription, a plasmid with a T7-promoter, dgcM ORF, a FLAG-tag, and T7-terminator was constructed (pET52-b_dgcM_FLAG) from the pET52-b vector (Novagen) backbone, already carrying a T7-promoter and terminator sequence. The plasmid was linearized by PCR using primers MHO-100/MHO-101, amplified with primers MHO-238 and MHO-239, introducing a FLAG-tag and T7-terminator restriction sites, and after digestion with both enzymes, ligated into DbaI/Nhel digested pXG-10. Plasmids pOmrA_M2 and pOmrB_M2 were created using inverse PCR mutagenesis, with the desired mutations included in the primers (MHO-221/MHO-223 for OmaR/OmrB, respectively, and MHO-222). Briefly, after PCR amplification with the phosphorylated primers, pOmrA or pOmrB template DNA was digested by DpnI. PCR product was purified (GeneJET PCR Purification Kit Therm Fischer Scientific) and religated with Ready-to-go T4 ligase (Amersham). The pDgcM_M2::3xFLAG plasmid was created with primers MHO-217 and MHO-218 on pDgcM::3xFLAG as template. To create a dgcM strain, dgcM was replaced by a FRT-flanked Amp R cassette by Lambda Red recombination (Yu et al., 2000) using primers MHO-094 and MHO-095. For dgcM transcription, a plasmid with a T7-promoter, dgcM ORF, a FLAG-tag, and T7-terminator was constructed (pET52-b_dgcM_FLAG) from the pET52-b vector (Novagen) backbone, already carrying a T7-promoter and terminator sequence. The plasmid was linearized by PCR using primers MHO-238 and MHO-239, introducing a FLAG-tag and T7-terminator restriction sites, and after digestion with both enzymes, vector and dgcM insert were ligated. E. coli Top10 cells were used for transformations (Invitrogen).

The csgD::M4 and ompR::mut2 mutations were introduced into strain MC4100 by Lambda red recombination (Yu et al., 2000) using plasmid pSm5-tet (Koskineni et al., 2011). First, a kan-sacB cassette (Pietsch et al., 2017), amplified with primers MHO-100/ MHO-101 for csgD::M4, and MHO-268/MHO-269 for ompR::mut2, was inserted at the site of mutagenesis. A second round of recombination was done with a ssDNA fragment carrying the desired point mutation, followed by counterselection for SacB (Ellis et al., 2001). To create the double mutant, ompR::mut2 was introduced into a wt as well as the csgD::M4 background.

The F42A (prox) and Y25D (dist) hfq mutant strains were previously published (Zhang et al., 2013; Appendix Table S2). The R168- R17A-R19A-K47E (rim) mutation, as well as an hfq deletion, was...
introduced into the chromosome of the same MC4100 relA− strain background. This was done by replacing the cat-sacB cassette in MC4100 relA− Δhfq::cat-sacB (Zhang et al., 2013), with a PCR fragment carrying the rim mutation, amplified from plasmid pETM60-St_Hfq102_R16S_R17A_R19A_K47E (Sauer et al., 2012) using primers MHO-294 and MHO-295, or with an oligo matching the hfq flanking region (MHO-291). The resulting strains were transformed with plasmids pDgcM::GFP and either pOmrA, pOmrB, or pControl.

Fluorescence measurements

For bulk measurements, bacterial cultures grown overnight from flanking region (MHO-291). The resulting strains were transformed St_Hfq102_R16S_R17A_R19A_K47E (Sauer et al., 2012) using primers MHO-294 and MHO-295, or with an oligo matching the hfq flanking region (MHO-291). The resulting strains were transformed with plasmids pDgcM::GFP and either pOmrA, pOmrB, or pControl.

Fluorescence measurements

For bulk measurements, bacterial cultures grown overnight from single colonies were diluted 1:100 in fresh LB medium and grown in 96-well black assay plates with clear flat bottom (Costar®) at 37°C. Fluorescence (GFP: excitation 480 nm, emission 520 nm) and optical density (600 nm) were measured for 23 h at 5-min intervals in a plate reader (Tecan Infinite Pro). Corrected fluorescence values were calculated as ratios between fluorescence and OD600. To account for cellular auto-fluorescence, GFP/OD600 from a strain not expressing a fluorescent protein was subtracted. Background-subtracted GFP/OD600 values from each strain and time point were averaged and expressed relative to the corresponding strain transformed with the control plasmid lacking an sRNA gene.

Single-cell analysis of GFP fluorescence was conducted by flow cytometry on a MACSQuant® YB instrument (Miltenyi Biotec). Cells were diluted in sterile-filtered PBS and loaded in a 96-well assay plate for analysis. GFP was excited with a blue laser (488 nm) in channel B1 (bandpass filter 525/50). For each sample, 100,000 events were recorded. The acquired data were processed with FlowJo software (FlowJo LLC) as follows. To exclude background noise, the events were first gated for bacterial cells based on side scatter measurements. Subsequently, the GFP fluorescence for the gated population was plotted as a histogram.

In vitro transcription of RNA

OmrA and OmrB DNA templates containing a T7 promoter, as well as a hammerhead ribozyme sequence, were generated as previously described (Holmqvist et al., 2010). OmrA M2 was generated similarly using primers MHO-248 and MHO-249. To generate a 210 nt dgcM cassette out of strain E388 (Holmqvist et al., 2010), OmrA_M2 was generated similarly using primers MHO-248 and MHO-249. To generate a 210 nt dgcM mRNA for structure probing and toeprinting assays, DNA templates containing a T7 promoter sequence were generated by PCR using primers MHO-159/MHO-194 with E. coli MC4100 relA− DNA as a template. The DNA templates were used for in vitro transcription using the Megascript Kit (Life Technologies, #AM1330). Transcription reactions were DNase I-treated (Roche) for 30 min at 37°C, followed by phenol–chloroform extraction and ethanol precipitation. RNAs were purified from denaturing polyacrylamide-urea gels by elution into 300 mM sodium acetate, 0.1% SDS, and 1 mM EDTA. After phenol–chloroform extraction, ethanol precipitation, washing with 80% ethanol, and drying, RNA was dissolved in TE buffer. RNA concentration and quality was assessed by NanoDrop and Denaturing PAGE.

5′ end-labeling was performed on CIAP-treated RNA (Invitrogen™, #18090-019) with T4 PNK (Thermo Fisher Scientific, #EK0831) and [γ-32P]ATP (10 mCi/ml, 3,000 Ci/mmole). Labeled RNAs were gel-purified as above. Before use, RNAs were reloaded in water for 1 min at 95°C followed by 1-min incubation on ice and renatured for 5 min at 37°C in renaturation buffer (100 mM K-acetate, 10 mM Mg-acetate, 50 mM HEPES-NaOH pH 7.5).

For in vitro translation assays, ompA and dgcM RNAs including C-terminal FLAG-tag and terminator sequences were used. PCR template for ompA-FLAG RNA transcription was generated using primers MHO-207 and MHO-230 from DNA of strain MC4100 ompA-FLAG. This strain was created by flipping the kanamycin resistance cassette out of strain E388 (Holmqvist et al., 2010) using the pCP20 plasmid. For dgcM transcription, the PCR fragment used contained the T7 promoter, the FLAG-tagged dgcM sequence, and a T7 terminator (template pET52b_dgcM_FLAG, primers MHO-244/MHO-245). Structural probing was carried out on 5′ end-labeled RNA. Reactions were performed at 37°C in the presence of 1 μg carrier yeast tRNA. When applicable, OmrA/B binding to dgcM RNA was allowed for 15 min in presence or absence of Hfq hexamers (50 nM). Enzymatic probing was done with 0.1 unit of RNase T1 (Invitrogen™, #AM22883) for 5 min, stopped by addition of cold Na-acetate (0.3 M), followed by phenol/chloroform/isoamyl alcohol (25/24/1) extraction. Chemical probing was done with lead(II) acetate at a final concentration of 5 mM for 5 min. Reactions were stopped with cold EDTA (50 mM final concentration). After precipitation, RNAs pellets were dried and dissolved in loading dye. Samples were resolved on 7.5% sequencing gels, fixed for 5 min (10% ethanol, 6% acetic acid), transferred to 3-mm Whatman paper, and dried. Signals were detected using a PhosphorImager screen and a PMI scanner™ (Bio-Rad).

Toeprinting assays

Final concentrations in 10 μl toeprinting reactions were 20 nM of mRNA and, when present, 100 nM of 30S subunits, 300 nM of initiator tRNA, and 0.5 mM of dNTPs. Briefly, mRNAs were denatured for 1 min at 90°C with radiolabeled primers and dNTPs in 1× RT-buffer (10 mM Tris acetate pH 7.6, 100 mM K-acetate, 1 mM DTT). After 1 min on ice, Mg-acetate was added to 10 mM final concentration. RNAs were refolded for 5 min, followed by addition of activated 30S (15 min at 37°C in 1× RT-buffer). After 10 min, initiator tRNA was added, and incubation continued for 25 min. Reverse transcription was started by addition of 100 U of SSIV (Invitrogen™, #18090010) for 20 min. Reactions were stopped by cold STOP buffer (50 mM Tris–HCl pH 7.5, 0.1% SDS, 10 mM EDTA). After phenol/chloroform extraction, the RNA template was removed by KOH treatment for 3 min at 95°C. After base neutralization with acetic acid, the cDNAs were ethanol-precipitated, centrifuged, dissolved in loading buffer, and resolved on 7.5% sequencing gels. Gels were fixed and treated as above.

Gel mobility shift assay

5′ end-labeled refolded RNA was incubated at various concentrations of unlabeled complementary RNA, in presence or absence of Hfq, for 30 min at 37°C. Reactions were stopped by adding loading dye (48% glycerol, 0.01% xylene cyanol, 0.01% bromophenol blue), and samples directly loaded on a 5% non-denaturing PAGE. To avoid heat-induced duplex separation, gels were run at 4°C for 3 h (300 V) in 0.5× TEB. Gel transfer and analysis was as
Cells were pelleted by centrifugation and frozen in liquid nitrogen. After thawing on ice, total RNA was isolated by the hot acid-phenol method (Blomberg et al., 1990). 5 µg of extracted total RNA was mixed with 2× RNA loading buffer [95% (v/v) formamide, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol], heated for 2 min at 95°C, and separated on an 8% sequencing gel. After electrophoresis (Bio-Rad Trans-Blot cell), RNA was transferred to an Amersham Hybond-C™-N+ membrane (GE Healthcare) by electro-blotting, and UV-crosslinked. 5'-end-labeled oligodeoxynucleotide probes were used for detection of Omra (EHO-406), Omrb (EHO-407) and, as loading control, SS rRNA (EHO-690). Prehybridization and hybridization of the membrane were carried out in modified Church and Gilbert hybridization buffer (Church & Gilbert, 1984), at 42°C. For visualization of bands, a Personal Molecular Imager (PMI—Bio-Rad) was used.

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**Author contributions**

MH and CR conducted the experimental work. MH, CR, EH, and EGHW planned the work, evaluated the data, and wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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The EMBO Journal e101199 | 2019 11 of 14
sRNA access needs mRNA remodeling by Hfq

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