Regulation of Hfq by the RNA CrcZ in *Pseudomonas aeruginosa* Carbon Catabolite Repression

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Abstract

Carbon Catabolite repression (CCR) allows a fast adaptation of Bacteria to changing nutrient supplies. The *Pseudomonas aeruginosa* (PAO1) catabolite repression control protein (Crc) was deemed to act as a translational repressor, repressing functions involved in uptake and utilization of carbon sources. However, Crc of PAO1 was recently shown to be devoid of RNA binding activity. In this study the RNA chaperone Hfq was identified as the principle post-transcriptional regulator of CCR in PAO1. Hfq is shown to bind to A-rich sequences within the ribosome binding site of the model mRNA amiE, and to repress translation *in vitro* and *in vivo*. We further report that Crc plays an unknown ancillary role, as full-fledged repression of amiE and other CCR-regulated mRNAs *in vivo* required its presence. Moreover, we show that the regulatory RNA CrcZ, transcription of which is augmented when CCR is alleviated, binds to Hfq with high affinity. This study on CCR in PAO1 revealed a novel concept for Hfq function, wherein the regulatory RNA CrcZ acts as a decoy to abrogate Hfq-mediated translational repression of catabolic genes and thus highlights the central role of RNA based regulation in CCR of PAO1.

Introduction

The opportunistic human pathogen *Pseudomonas aeruginosa* causes acute as well as chronic infections in immunocompromised individuals. Moreover, airway epithelia of patients suffering from cystic fibrosis are frequently colonized by the pathogen [1]. *P. aeruginosa* is a metabolically versatile organism with the ability to utilize numerous carbon sources, which allows the bacterium to thrive in different environments such as soil, marine habitats as well as on/in different organisms [2].

In Bacteria, the uptake and utilization of carbon compounds is controlled in a hierarchical manner by a mechanism known as carbon catabolite repression (CCR). Generally speaking, CCR prevents the utilization of less preferred carbon sources until the preferred one is consumed. In *Escherichia coli* CCR prevents the expression of catabolic genes, the transcription of which requires the transcriptional activator CRP (cyclic AMP receptor protein) in conjunction with cAMP, whereas in *Bacillus subtilis* CCR is mediated by the transcriptional repressor CcpA (catabolite control protein A). In both organisms CCR is regulated by a signal transduction pathway intrinsic to the phosphoenolpyruvate-carbohydrate phosphotransferase system [3].

In most studied *Pseudomonas* spp. the presence of organic acids (for example succinate) results in CCR, which leads to repression of catabolic genes required for the consumption of other carbon sources. During CCR catabolic genes were deemed to be downregulated by the translation repressor Crc (*catabolite repression control protein*) [4]. It has been suggested that Crc binds to C-rich motifs within or adjacent to ribosome binding sites (RBS) of multiple target mRNAs, and thereby prevents their translation [5–7]. Upon relief of CCR, the regulatory RNAs, CrcZ in PAO1 [7], CrcZ/CrcY in *P. putida* [8] and CrcZ/CrcX in *P. syringae* [9] were proposed to bind to and to counteract Crc by trapping the protein. This hypothesis was in line with the observation that the CrcZ levels increase in the presence of poor carbon sources, and that they are reduced in the presence of a preferred carbon source [7]. However, our recent structural and biochemical studies challenged the role of Crc as a direct translational repressor of genes governed by CCR in PAO1. Recombinant Crc purified to homogeneity did neither bind to *amiE* mRNA, encoding aliphatic amidase, nor to CrcZ RNA [10,11]. Rather, the previously reported RNA binding activity of His-tagged Crc purified by nickel affinity chromatography [6,7] was attributed to a contamination of the Crc-His preparations with the RNA chaperone Hfq [10,11].

In *Enterobacteriaceae* Hfq is pivotal for riboregulation [12,13], which results on the one hand from binding to and protection of sRNAs from nucleolytic decay [14], and on the other hand from accelerating base-pairing between sRNAs and their target mRNAs [15–17]. *E. coli* Hfq hexamers have dedicated RNA binding sites, preferably binding uridine-rich stretches of sRNAs around the central pore of the proximal surface [18,19] and A-rich sequences on the distal surface [20]. In addition, the lateral surface of the hexamer can as well contribute to sRNA binding [21]. The dedicated sRNA and mRNA binding surfaces on either site of the Hfq-hexamer may serve to transiently increase the local concentration of two RNA substrates. Moreover, the inherent capacity of Hfq to induce conformational changes in RNAs together with the observed structural flexibility of RNA ligands bound to Hfq could stochastically facilitate base-pairing [22,23].
Carbon assimilation in Bacteria is governed by a mechanism known as carbon catabolite repression (CCR). In contrast to several other bacterial clades CCR in Pseudomonas species appears to be primarily regulated at the post-transcriptional level. In this study, we have identified the RNA chaperone Hfq as the principle post-transcriptional regulator of CCR in P. aeruginosa (PAO1). Hfq is shown to act as a translational regulator and to prevent ribosome loading through binding to A-rich sequences within the ribosome binding site of mRNAs, which encode enzymes involved in carbon utilization. It has been previously shown that the synthesis of the RNA CrcZ is augmented in the presence of non-preferred carbon sources. Here, we show that the CrcZ RNA binds to and sequesters Hfq, which in turn abrogates Hfq-mediated translational repression of mRNAs, the encoded functions of which are required for the breakdown of non-preferred carbon sources. This novel mechanistic twist on Hfq function not only highlights the central role of RNA based regulation in CCR of PAO1 but also broadens the view of Hfq-mediated post-transcriptional mechanisms.

Author Summary

Carbon assimilation in Bacteria is governed by a mechanism known as carbon catabolite repression (CCR). In contrast to several other bacterial clades CCR in Pseudomonas species appears to be primarily regulated at the post-transcriptional level. In this study, we have identified the RNA chaperone Hfq as the principle post-transcriptional regulator of CCR in P. aeruginosa (PAO1). Hfq is shown to act as a translational regulator and to prevent ribosome loading through binding to A-rich sequences within the ribosome binding site of mRNAs, which encode enzymes involved in carbon utilization. It has been previously shown that the synthesis of the RNA CrcZ is augmented in the presence of non-preferred carbon sources. Here, we show that the CrcZ RNA binds to and sequesters Hfq, which in turn abrogates Hfq-mediated translational repression of mRNAs, the encoded functions of which are required for the breakdown of non-preferred carbon sources. This novel mechanistic twist on Hfq function not only highlights the central role of RNA based regulation in CCR of PAO1 but also broadens the view of Hfq-mediated post-transcriptional mechanisms.

Although many sRNA candidates have been identified in PAO1 [24–27], the function of only a few has been revealed. The sRNAs PhrS [28] and PrrF [29] have been shown and inferred, respectively, to act by base-pairing with target mRNAs, whereas the protein binding RNAs RsmY and RsmZ are known to antagonize the function of the translational regulator RsmA [30]. PAO1 Hfq was shown to stabilize the protein binding RNA RsmY [31,32] and to affect expression of some sRNAs including PhrS [25]. In PAO1, Hfq acts as a pleiotropic regulator, impacting on growth, virulence, motility, and quorum sensing [31,33]. A transcriptome analysis of a PAO1 Δhfq- strain revealed that ~15% of all genes were de-regulated. These included a number of genes encoding proteins involved in carbon compound catabolism, which were up-regulated in the absence of Hfq [31].

Here, we studied the impact of Hfq on CCR in PAO1. In vivo and in vitro studies revealed that Hfq acts as a translational repressor of several catabolic genes. Moreover, we present evidence that the regulatory RNA CrcZ binds to and sequesters Hfq, which in turn results in translation of Hfq-regulated mRNAs. Hence, this study revealed a novel mechanistic twist on post-transcriptional regulation by Hfq and highlights its role in regulating the central metabolism in P. aeruginosa.

Results

Hfq represses catabolic genes at the post-transcriptional level

Several observations suggested a link between Hfq and CCR in PAO1. A comparative transcriptome analysis of a PAO1 wt and a PAO1 Δhfq- strain disclosed transcripts encoding functions related to carbon compound and amino-acid catabolism that were up-regulated in the absence of Hfq [31; Table S1]. Many of these transcripts comprise A-rich stretches (Table S1) within or adjacent to the RBS, which could serve as recognition motifs for the distal poly-A binding site of Hfq [20,34]. Mutations within these A-rich stretches in certain mRNAs abrogated CCR [7,33].

Crc was deemed to act as a translational regulator in PAO1 CCR [5–7]. However, Crc was recently shown to be deficient in binding to CrcZ and to the CCR regulated amiE mRNA [10]. In fact, these studies identified Hfq as a contaminant RNA binding activity in Crc preparations [10,11]. This observation prompted us to test (i) whether Hfq serves as the principle post-transcriptional regulator of CCR in PAO1, and (ii) whether the regulatory RNA CrcZ, displaying several A-rich motifs [7], might abrogate Hfq-mediated regulation by sequestering Hfq.

We first revisited post-transcriptional regulation of amiE mRNA, encoding an aliphatic amidase. Strain PAO1(pTCamiE) and strain PAO1(pME9655) harboring a plasmid borned transcriptional amiE-lacZ fusion, and a translational amiE-lacZ fusion, respectively, were grown in BSM medium either in the presence of succinate/acetamide (CCR) or mannitol/acetamide (no CCR). Acetamide was added to induce transcription of the chimeric amiE genes, i.e. to mimic CCR. As shown in Figure S1A (left panel), the β-galactosidase activities conferred by the translational fusion was comparable in either medium, i.e. in the presence and absence of CCR. However, when compared with growth in the presence of mannitol/acetamide (no CCR), amiE-lacZ translation was repressed in strain PAO1(pME9655) (Figure S1A; right panel) during cultivation in the presence of succinate/acetamide, i.e. when CCR was in place.

We next tested whether amiE mRNA and CrcZ RNA associate with Hfq upon induction of amiE transcription during CCR. Strain PAO1Δhfq- harboring plasmid pMMBbhfq15 (encodes flag-tagged Hfq) and the control plasmid pMMB67HE, respectively, was grown to an OD600 of 1.5 in BSM medium supplemented with succinate. In addition, acetamide was added to induce transcription of the amiE gene, i.e. to mimic CCR. Then, cell lysates were prepared and Hfq-associated RNAs were co-immunoprecipitated (CoIP) with Hfq-specific antibodies. As revealed by RT-PCR both, amiE and CrcZ, were found in complex with Hfq (Figure S2). In contrast RsmZ, which does not bind to Hfq [7], could not be detected among the RNAs that co-immunoprecipitated with Hfq (Figure S2). Taken together, these initial studies validated amiE as a model mRNA to scrutinize the hypothesized role of Hfq in CCR.

To obtain first hints whether Hfq is involved in post-transcriptional regulation of amiE during CCR, the strains PAO1, PAO1 Δcrc and PAO1 Δcrc were transformed with plasmid pTCamiE harboring the translational amiE-lacZ fusion and with plasmid pME9655 harboring the translational amiE-lacZ fusion, respectively. The strains were grown in BSM medium in the presence of succinate to establish CCR, and in the presence of acetamide to induce amiE-lacZ/amiE-lacZ transcription. The β-galactosidase activity conferred by the translational amiE-lacZ fusion was comparable in the presence and absence of Hfq and/or Crc in either strain (Figure S1B). In contrast, the β-galactosidase activity conferred by the translational amiE-lacZ fusion differed in strains PAO1, PAO1 Δhfq- and PAO1 Δhfq- Δcrc when grown in BSM medium containing succinate and acetamide (CCR). In contrast to PAO1, amiE-lacZ translation was greatly increased in the PAO1 Δhfq- strain and in the PAO1 Δhfq- Δcrc double mutant, respectively (Figure 1A). The absence of Crc resulted as well in marked amiE-lacZ translation, albeit at a lower level when compared with the Δhfq- mutant or the Δhfq- Δcrc double mutant, suggesting that Hfq exerts a more pronounced negative effect on amiE translation during CCR than Crc. To verify these experiments, we tested whether Hfq likewise affects translation of the estA and the phbM genes, which are also known to be regulated by CCR [7,35,36]. The impact of Hfq was again monitored using translational lacZ reporter gene fusions. The results obtained mirrored those obtained with the amiE-lacZ reporter gene. Their translation was repressed during growth of PAO1 in BSM medium containing succinate, whereas in the absence of Hfq, Crc or both, the synthesis of the encoded fusion proteins increased (Figures 1B
Figure 1. Repression of catabolic genes by Hfq. (A–C) The strains were grown to an OD₆₀₀ of 2.0 in BSM medium supplemented with (A) 40 mM succinate and 40 mM acetamide (to establish CCR and to induce amiE::lacZ transcription) or (B, C) only with 40 mM succinate (CCR). Then, the cells were harvested and the β-galactosidase activities were determined. The bars depict β-galactosidase values conferred by the translational amiE::lacZ fusion encoded by plasmid pME9655 (A), the estA::lacZ fusion encoded by plasmid pTLEstA (B) and the phzM::lacZ fusion encoded by plasmid pME10011 (C) in strains PAO1 (blue bar), PAO1Δcrc (green bar), PAO1Δhfq (red bar) and PAO1Δhfq-Δcrc (purple bar), respectively. The error bars represent standard deviations from three independent experiments. (D, E) The strains were grown in BSM medium supplemented with succinate and acetamide. At an OD₆₀₀ of 1.0 IPTG (1 mM final concentration) was added to strains PAO1Δcrc(pMMBcrcFlag; pME9655) (blue bar), PAO1Δhfq-Δcrc(pMMBhfqFlag; pME9655) (red bar) and PAO1Δhfq-Δcrc(pMMBhfqFlag; pME9655) (purple bar), respectively. The bars represent the β-galactosidase values conferred by the plasmid pME9655 encoded translational amiE::lacZ fusion 3 h after induction of the plasmid borne crcFlag gene. The error bars represent standard deviations from three independent experiments. The corresponding CrcFlag levels were determined by western-blot analysis using anti-Flag antibodies in strains PAO1Δcrc(pMMBcrcFlag; pME9655), PAO1Δhfq-Δcrc(pMMBhfqFlag; pME9655) and PAO1Δhfq-Δcrc(pMMBhfqFlag; pME9655). Immunodetection of ribosomal protein S2 (loading control) was performed as described in Materials and Methods. (E) At an OD₆₀₀ of 1.0 IPTG (1 mM final concentration) was added to strains PAO1Δhfq-Δcrc(pMMBhfqFlag; pME9655) (blue bar), PAO1Δhfq-Δcrc(pMMBhfqFlag; pME9655) (green bar) and PAO1Δhfq-Δcrc(pMMBhfqFlag; pME9655) (purple bar), respectively. The bars represent the β-galactosidase values conferred by the plasmid pME9655 encoded translational amiE::lacZ fusion 3 h after hfqFlag induction. The error bars represent standard deviations from three independent experiments. The corresponding HfqFlag levels were determined by western-blot analysis using anti-Flag antibodies in strains PAO1Δhfq-Δcrc(pMMBhfqFlag; pME9655), PAO1Δhfq-Δcrc(pMMBhfqFlag; pME9655) and PAO1Δhfq-Δcrc(pMMBhfqFlag; pME9655) (top panel). Immunodetection of ribosomal protein S2 (loading control) was performed as described in Materials and Methods.

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In contrast, the expression of the heterologous lacZ gene (variation control), was comparable in strains PAO1, PAO1\(^{hfq^{-}}\), PAO1\(^{Dcrc}\) and PAO1\(^{hfq^{-}}Dcrc\) (Figure S1C). Taken together, these initial studies supported our hypothesis that Hfq is involved in post-transcriptional regulation of CCR regulated genes.

Hfq acts as the principle post-transcriptional regulator and Crc has an auxiliary function in CCR

As shown in Figure 1A–C, both, Crc and Hfq, were required for full repression of all three CCR regulated genes. Although translation occurred in the absence of Crc, a pronounced increase in translation only required the absence of Hfq, i.e. the observed de-repression was comparable in the hfq\(^{-}\) strain and in the hfq\(^{-}Dcrc\) double mutant. We interpreted this as showing that Hfq acts as the principal translational repressor, whereas Crc seemed to act as an auxiliary factor, somehow amplifying the negative regulation exerted by Hfq. To further test this hypothesis, amiE::lacZ translation was monitored in the PAO1\(^{hfq^{-}}Dcrc\) double mutant complemented with a plasmid borne crc\(^{Flag}\) and hfq\(^{Flag}\) gene, respectively. In consideration that crc could impinge on hfq expression and vice versa, the plasmid pMMB\(^{crc^{Flag}}\) and pMMB\(^{hfq^{Flag}}\) borne \(\alpha_{1^{Flag}}\) and \(h_{f}^{Flag}\) genes, respectively, were equipped with the same expression signals, i.e. their expression was controlled by the \(P_{sa}\) promoter and identical translation initiation signals. The different strains used in this experiment were grown in BSM medium supplemented with succinate and acetamide (CCR). At an OD\(_{600}\) of 1.0, IPTG was added to induce ectopic expression of the \(\alpha_{1^{Flag}}\) and \(h_{f}^{Flag}\) genes, respectively. Three hours thereafter,
amiE::lacZ translation was monitored by determination of the β-galactosidase activities and the Crc-Flag and Hfq-Flag levels were determined by quantitative western-blot analysis.

As shown in Figure 1D (blue bar), under these conditions amiE::lacZ translation was repressed in strain PAO1Δcrc(pMMB67HE; pME9655). In contrast, translation of the amiE::lacZ fusion gene was derepressed in the absence of Hfq in the double mutant PAO1ΔhfqΔcrc(pMMB67HE; pME9655) (Figure 1D; red bar). Moreover, when compared with the control strain PAO1ΔhfqΔcrc(pMMB67HE; pME9655) (Figure 1D; purple bar), ectopic expression of crfFlag in strain PAO1ΔhfqΔcrc(pMMB67HE; pME9655) did not affect amiE::lacZ translation in the absence of Hfq.

Next, amiE::lacZ translation was monitored in strains PAO1Δhfq−Δcrc(pMMB67HE; pME9655) and PAO1Δhfq−Δcrc(pMMB67HE; pME9655) after growth in BSM succinate/acetamide medium (CCR) and 3 h after ectopic expression of the hfqFlag gene. The absence of Crc in the double mutant strain PAO1Δhfq−Δcrc(pMMB67HE; pME9655) (Figure 1D; green bar) resulted in an increased de-repression of amiE::lacZ translation when compared with strain PAO1Δhfq−Δcrc(pMMB67HE; pME9655) (Figure 1D; blue bar) despite comparable levels of HfqFlag in both strains. Taken together, these experiments showed that Crc only impacts on amiE::lacZ translation in the presence of Hfq. Hence, they corroborate the idea that Crc does not act per se as a translational regulator but functions as an ancillary factor in Hfq-mediated repression of target genes.

Hfq binds to the translation initiation region of amiE mRNA and prevents in vitro ribosome binding and translation

Next, we tested whether Hfq directly represses translation of amiE mRNA by binding to the translation initiation region (TIR). First, a filter binding assay was performed with purified PAO1 Hfq and an amiE mRNA fragment encompassing nucleotides (nt) from position −134 to +20 with regard to the A (+1) of the start codon. This experiment revealed that Hfq binds to amiE−134+20 with a Kd of ~67.0±1.4 nM (Figure S3). Next, the Hfq binding site(s) were mapped on amiE RNA. Enzymatic probing was performed with riboendonucleases T1 (G-specific cleavage) and A (C/U-specific cleavage) in the absence and presence of Hfq. As shown in Figure 2, Hfq protected the segment of amiE RNA extending from G−281 to U−5. This region includes the Shine and Dalgarno sequence (SD) sequence of amiE mRNA. Thus, Hfq binding to this region would readily explain the observed translational repression of amiE::lacZ mRNA (Figure 1A, D, E).

To further test whether Hfq acts as a translational repressor of amiE mRNA, the PURExpress system was employed. The in vitro translation system was programmed with amiEFlag mRNA, encoding a Flag-tagged amidase. As shown in Figure S4, translation of amiEFlag mRNA was already impeded at a 1:1 molar ratio of Hfq to mRNA. As the in vitro system is reconstituted from purified components of the translation machinery of E. coli, no additional PAO1 component was apparently required for repression.

To further demonstrate that Hfq directly interferes with ribosome binding, a toeprinting assay was performed with amiE−134+76 mRNA in the presence and absence of Hfq. Briefly, in the presence of tRNA\textsuperscript{Met}, 3OS ribosomes form a stable ternary complex at the RBS of mRNAs, which can be visualized by inhibition of cdNA synthesis primed downstream of the start codon [37]. A toeprint signal usually occurs at position +15 to +17 with regard to the A (+1) of the start codon. As shown in Figure 3, lane 7, a toeprint signal at the amiE RBS was observed in the absence of Hfq whereas the addition of Hfq to amiE−134+76
mRNA inhibited ternary complex formation (Figure 3, lanes 8 and 9). In contrast, the presence of Hfq alone did not result in a stop signal (Figure 3, lane 6), which is in accordance with earlier observations that translational repressors do not always provide a roadblock to cDNA synthesis by reverse transcriptase under these conditions [38]. Taken together, these in vitro studies strongly supported the idea that Hfq acts as a translational repressor that prevents ribosome loading on amiE mRNA.

The distal site of Hfq is required for regulation of amiE mRNA

Link et al. [20] reported a crystal structure of E. coli Hfq in complex with poly(A)_15, wherein the poly(A) tract is bound to the distal face using tripartite binding motifs. They consist of an adenosine specific site (A-site), a purine nucleotide selectivity site (R-site) and a sequence-non-discriminating E-site. The amino-acids involved in building the A-R-N motifs are fully conserved in the Hfq protein of PAO1 [34]. As the hexamer Hfq could accommodate the entire A-rich stretch from nt −26 to −8 of amiE mRNA (Figure 2) in the six binding pockets, we next tested whether the distal binding site of Hfq is required and sufficient for translational repression of amiE mRNA. We therefore engineered the PAO1 hfq variant hffK56AFlag and hffY25DFlag as the corresponding E. coli mutant proteins were shown to be deficient in binding to polyA- and polyU-tracks [18], respectively. In contrast to the PAO1 hffK56AFlag gene and the PAO1 hffY25DFlag allele, ectopic expression of the hffY25DFlag allele did not result in repression of amiE::lacZ translation, albeit all three proteins, HffK56AFlag, HffY25DFlag and HffK56AFlag were present at comparable levels (Figure 4A). Basically the same results were obtained when translation of the estA::lacZ (Figure 4B) and phzM::lacZ (Figure 4C) fusion genes was monitored in the presence of HffK56AFlag, HffY25DFlag and HffK56AFlag, respectively. Hence, translational repression of these reporter genes apparently required an intact distal polyA binding site of Hfq.

To verify these in vivo data, the PAO1 Hfq variants HffY25D and HffK56A were purified, and binding to amiE−134+20 mRNA was assessed using electrophoretic mobility shift assays (EMSA). As shown in Figure 5A, while PAO1 Hff and HffK56A bound to the mRNA fragment, the HffY25D protein failed to bind. With increasing concentrations of either Hff or HffK56A two shifted bands were observed (Figure 5A), suggesting that Hff binds at two sites of the amiE−134+20 fragment. This observation can be explained by the Hff binding site mapped between nucleotides G−28 to U−5 (Figure 2) and by a probable second binding site, comprising an A-rich region (nucleotides −92 to −71), which was inferred from an RNomics approach after CoIP with Hff-specific antibodies [25]. Taken the in vivo and in vitro studies together, these experiments strongly suggested that Hff binds with its distal face to the RBS of amiE, and by inference most likely also to estA and phzM mRNA.

To further corroborate the idea that Hff directly represses amiE translation the experiment was also performed in the heterologous E. coli hfq− strain JW4130. The strain was transformed with the control plasmid pME4510 and derivatives thereof harboring the PAO1 hffFlag gene, the PAO1 hffY25DFlag allele and the PAO1 hffK56AFlag allele, respectively. In addition, these strains were transformed with plasmid pME9658, wherein the amiL terminator preceding the amiE gene was deleted. As shown in Figure 5B, the experimental results paralleled that performed in PAO1. The translation of the amiE::lacZ gene was repressed in the presence of HffFlag and HffK56AFlag whereas the reporter gene was translated in the presence of HffY25DFlag. Thus, the presence of Hff was apparently necessary and sufficient for repression of amiE translation in E. coli.

The regulatory RNA CrcZ titrates Hff in vitro

The regulatory RNA CrcZ is present at lower levels during CCR, when compared to conditions when CCR is not in place, e.g. in BSM medium containing mannitol as the sole carbon source [7]. The 407 nt long CrcZ RNA contains six A-rich stretches (Figure S6A) to which Hff can potentially bind with its distal face, i.e. with the same binding surface as it binds to the RBS of amiE (Figure S5A). Binding of Hff to the first 151 nt of CrcZ, containing 3 A-rich stretches (Figure S6A) was confirmed using EMSA assays (Figure S6B). Three shifted bands could be discerned (Figure S6B, lane 2), which would be consistent with one Hff-hexamer binding to either A-rich stretch. As anticipated, the
can abrogate Hfq-mediated translational repression in vitro

The absence of CCR (see Figure S1A). 2–7, Hfq-hexamer was added in 20-fold molar excess over labeled unlabelled CrcZ151 RNA and unlabelled RsmZ RNA were added, respectively. The molar ratios of the competitor RNAs to doi:10.1371/journal.pgen.1004440.g005

inhibition of on top. Lanes 5–7, Hfq was added in 2-fold molar excess over Hfq. Lane 7, RsmZ was added in 2-fold molar excess over amiE

Figure 5. CrcZ competes with amiE for Hfq. (A) Electrophoretic mobility shift assay with 10 nM radioactively labeled amiE−134−20 mRNA in the presence of Hfq protein and unlabelled specific or non-specific competitor RNAs. Lane 1, no protein was added to labeled amiE−134−20 mRNA. Lane 2–7, Hfq-hexamer was added in 20-fold molar excess over labeled amiE−134−20 RNA. Lanes 3 and 4, 5 and 6, and 7, unlabeled amiE−134−20 RNA, unlabelled CrcZ151 RNA and unlabelled RsmZ RNA were added, respectively. The molar ratios of the competitor RNAs to amiE−134−20 mRNA are shown on top. (B) Repression of amiEFlag mRNA translation is relieved in the presence of CrcZ. Lane 1, in vitro translation of amiEFlag mRNA. Lanes 2–4, inhibition of amiEFlag mRNA translation in the presence of increasing amounts of Hfq. The molar ratios of Hfq-hexamer to amiEFlag mRNA are denoted on top. Lanes 5–7, Hfq was added in 2-fold molar excess over amiEFlag mRNA. CrcZ was concomitantly added in equimolar concentration (lane 5) or in two-fold molar excess (lane 6) over Hfq. Lane 7, RsmZ was added in 2-fold molar excess over amiEFlag mRNA. doi:10.1371/journal.pgen.1004440.g005

PAO1 HfqV25D mutant protein was defective in binding to CrcZ151, whereas the HfqK56A variant bound to the CrcZ fragment like native Hfq. Thus, CrcZ binds to the distal face of Hfq like amiE mRNA, and therefore has the potential to titrate Hfq, which in turn would explain the observed increase in translation of amiE::lacZ mRNA in BSM mannitol medium, i.e. in the absence of CCR (see Figure S1A).

In the next set of experiments we therefore asked whether CrcZ can abrogate Hfq-mediated translational repression in vitro. First, a mobility shift assay was performed with radioactively labeled amiE−134−20 RNA in the presence of unlabelled specific CrcZ151 and non-specific RsmZ competitor RNAs, respectively. As shown in Figure 5A, CrcZ151 competed with amiE−134−20 for binding to Hfq (Figure 5A, lanes 5 and 6), whereas the addition of RsmZ RNA, which does not bind to Hfq [7], did not result in a downshift of labeled amiE−134−20 RNA (Figure 5A, lane 7). When compared with the addition of unlabelled amiE−134−20 RNA, the addition of an equimolar concentration of CrcZ151 resulted already in a significant downshift, i.e. loss of Hfq binding to amiE−134−20. Hence, CrcZ151 acted as a better competitor for Hfq than amiE−134−20, which is anticipated as the used CrcZ151 has three Hfq binding sites, whereas the amiE fragment has only two.

Next, amiEFlag mRNA was translated in vitro in the presence of Hfq as well as in the presence of Hfq and CrcZ mRNA. As shown before (Figure S4), Hfq inhibited translation of amiEFlag (Figure 5B, lanes 2–4). In contrast, the mRNA was translated in the presence of CrcZ (Figure 5B, lanes 5 and 6), whereas the Hfq-mediated translational repression was not relieved in the presence of the non-specific competitor RNA RsmZ (Figure 5B, lane 7).

Similarly, the addition of CrcZ RNA to the Hfq-amiE complex, under conditions that inhibited translation initiation complex formation, resulted in reappearance of a toeprint signal (Figure 3, lanes 10 and 11). Thus, CrcZ counteracted the function of Hfq. Taken together, these in vitro experiments indicated that CrcZ can titrate Hfq, and that it can prevent it from binding to the amiE RBS.

CrcZ-mediated regulation of catabolic genes in vivo depends on Hfq

In our model titration of Hfq by CrcZ would result from increased CrcZ levels [7] in the presence of non-preferred carbon sources, which in turn would lead to de-repression of Hfq-regulated genes. Hence, it would be anticipated that ectopic over-expression of CrcZ would cause de-repression of Hfq-regulated genes even during CCR, i.e. under conditions when the CrcZ levels are low [7]. With this line of reasoning, we next asked whether ectopic expression of crcZ during CCR results in increased expression of the estE gene, which was assessed by monitoring the esterase activity.

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To avoid any interference of Crc, the experiments were performed in a PAO1Δcrc strain. The control strain PAO1Δcrc(pMMB67HE) and the crcZ over-expressing strain PAO1Δcrc(pMMBcreZ) were grown in BSM medium supplemented with 40 mM succinate to an OD590 of 1. Then, crcZ expression was induced and samples were taken 60 minutes thereafter. Esterase was produced even without induction of crcZ, which can be reconciled with the absence of Crc (see Figure 1B) and by endogenous background expression of crcZ in strain PAO1Δcrc(pMMB67HE) (Figure 6A, upper panel). Nevertheless, ectopic over-expression of crcZ (Figure 6A, upper panel) resulted in

Figure 6. CrcZ over-expression during CCR results in increased expression of estE. (A) The strain PAO1Δcrc(pMMB67HE) and the crcZ over-expressing strain PAO1Δcrc(pMMBcreZ) were grown in BSM medium supplemented with 40 mM succinate to an OD590 of 1. Then, crcZ expression was induced and samples were taken 60 minutes thereafter. Esterase was produced even without induction of crcZ, which can be reconciled with the absence of Crc (see Figure 1B) and by endogenous background expression of crcZ in strain PAO1Δcrc(pMMB67HE) (Figure 6A, upper panel). Nevertheless, ectopic over-expression of crcZ (Figure 6A, upper panel) resulted in

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abrogates Hfq-mediated translational repression. Support to the hypothesis that CrcZ titrates Hfq, and thereby the presence of Hfq. Hence, these in vivo amiE::lacZ experiments showed that the hfq- mutant is epistatic to the double mutant PAO1(hfq-). Taken together, these experiments lend further support to the hypothesis that CrcZ titrates Hfq, and thereby abrogates Hfq-mediated translational repression.

**Discussion**

**Target genes of Hfq in PAO1**

In this study, we provided evidence that Hfq represses three CCR regulated genes, amiE, estA and phzM. Given that Hfq blocked translation initiation of amiE mRNA by binding to A-rich stretches encompassing the RBS, we revisited catabolic and transport genes that were found to be up-regulated in a comparative transcriptome analysis of a PAO1 and a PAO1Δhfq mutant [31]. Out of 126 putative target mRNAs, 72 transcripts contained A-rich stretches in the TIR (Table S1). Out of the latter, 28 transcripts contain several single non-consecutive A-R-N motifs, whereas 44 transcripts contained consecutive (A-R-N)

Hfq in **Pseudomonas aeruginosa**: regulating a few at a time

Based on our *in vivo* and *in vitro* studies with the amiE model mRNA, we suggest that Hfq acts as a translational repressor of transcripts subjected to CCR in PAO1 (Figure 7). As there appear to be several Hfq regulated genes (Table S1) and our preliminary results show that Hfq levels are more or less constant during growth in different carbon sources (E. Sonnleitner, unpublished), the question arises how Hfq can regulate numerous mRNAs. Many if not all catabolic genes including the corresponding transporter genes are primarily regulated at the transcriptional level [4,40], and their transcription usually requires the presence of the respective catabolite. Therefore, it is rather unlikely that many of the putative target genes (Table S1) are concomitantly induced at the same time. Thus, in the presence of a preferred carbon sources.
source, e.g. succinate, only a few other catabolites may induce concomitant transcription of the corresponding catabolic genes. Moreover, translational repression most likely leads to degradation of mRNAs encoding catabolic enzymes other than those required for the breakdown of succinate, and thus to recycling of Hfq. Therefore, CCR control may not require vast amounts of Hfq. This hypothesis is supported by the experiment shown in Figure S8. When compared with the absence of CCR (presence of acetamide only) or with the PAO1Δhfq strain, amiE mRNA was faster degraded in PAO1 during CCR in the presence of succinate and acetamide. In addition, we have estimated the number of Hfq hexamers/cell in BSM- succinate medium with 2160+/−256 per PAO1 cell (Figure S9), which might suffice to silence “a few catabolic transcripts” that are induced during CCR (Figure 7).

In the presence of non-preferred carbon sources, the two component system CbrAB is activated; phosphorylated CbrB binds to the RpoN-dependent promoter of crcZ and stimulates CrcZ synthesis [7,43]. Our data (Figures 5 and 6) suggest that CrcZ then binds to and titrates Hfq by virtue of its A-rich binding motifs. This in turn would allow translation of transcripts that are induced by available catabolites, and thus synthesis of the cognate degradative enzymes (for example aliphatic amidase) (Figure 7).

For the following reasons we favor the hypothesis that no other sRNAs are required for regulation of the catabolic genes examined in this study. Most E. coli sRNAs bind to the proximal site of Hfq, and binding is usually abrogated in the HfqK56A mutant protein [13]. However, in contrast to the HfqY25D variant, the HfqK56A mutant protein was still capable to repress the fusion genes governed by CCR in PAO1 (Figure 4) and in E. coli (Figure S5B). It seems therefore reasonable to suggest that regulatory sRNAs other than CrcZ are not involved in Hfq-mediated regulation of these catabolic genes.

A novel concept for Hfq function: from mediating riboregulation to being regulated

Although binding of E. coli Hfq to A-rich stretches in mRNA has been demonstrated in several model systems [see below], Hfq seems to work predominantly in conjunction with sRNAs [44–46]. Hfq-mRNA binding may recruit the sRNA to the target mRNA and stimulate sRNA-mRNA pairing. In this scenario, the sRNA competes with initiating 30S ribosomes, whereas Hfq has a rather indirect function.

Some deviations from the canonical model of Hfq assisted and sRNA-mediated regulation of target mRNAs have been reported.

**Figure 7. Simplified model for CCR in Pseudomonas aeruginosa orchestrated by Hfq and CrcZ.** During CCR (left), for example during growth in the presence of succinate, the regulatory RNA CrcZ (in purple) is poorly transcribed. Upon catabolite-dependent induction of catabolic genes, for example induction of amiE transcription by acetamide, Hfq (red hexamer) represses translation of the corresponding transcripts, which in turn results in their degradation. When CCR is alleviated (right) the abundance of CrcZ increases. CrcZ binds to and titrates Hfq, which permits translation of catabolic genes, for example amiE.

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In *E. coli* evidence has been provided that Hfq acts as an autogenous repressor on its own mRNA. As for PAO1 amiE mRNA (Figure 2), *E. coli* Hfq was shown to bind to an A-rich sequence encompassing the SD sequence of *hfp* mRNA [47]. Another example for translational repression by Hfq entails regulation of the *E. coli* sdhC mRNA by the sRNA Spot 42. Desnoyers and Masse [48] reported that Spot 42 binds upstream of the RBS and recruits Hfq, which then directly represses translation. Moreover, *E. coli* Hfq was recently shown to act as a repressor of *cirA* mRNA translation in the absence of a sRNA [49]. Interestingly, the translational block exerted by Hfq was shown to be abrogated by RyhB RNA pairing to *cirA* mRNA [49].

Similarly, the model shown in Figure 7 entails direct repression of target mRNAs by PAO1 Hfq. However, in contrast to Spot 42 [48] and RyhB [49] RNA, which recruit Hfq to and abrogate Hfq-mediated translational repression of the target mRNA, respectively, CrcZ RNA acts as a decoy for Hfq. The CrcZ RNA contains six (A-R-N)$_n$ repeats ($n \geq 4$) (Figure S6A) that can potentially be exploited for binding to the distal face of Hfq. Thus, in the absence of CCR Hfq is most likely sequestered by CrcZ, and therefore not available to act as a translational repressor on target mRNAs. In this way the Hfq/CrcZ regulatory system is reminiscent to the *Pseudomonas* RsmA/RsmY/RsmZ system and the CsrA/CsrB system of several Gram-negative Bacteria [30,50,51]. The ability of Hfq to bind to RsmY [31] in fact poses the question whether the regulatory systems are interlinked. This could provide an explanation why different carbon sources can affect virulence traits such as biofilm formation and antibiotic resistance [39,52].

Moreover, given its constellation with Hfq, it seems possible that CrcZ on the one hand can interfere with any mRNA-dependent role of Hfq. On the other hand, the role of Hfq in sRNA-mediated riboregulation remains ill defined in PAO1. Although Hfq appears to be required for the PAO1 sRNAs PrfF1/PrfF2 to regulate target mRNAs [53], it is unclear whether Hfq exerts this function by stabilizing the sRNAs and/or by stimulating sRNA-mRNA pairing. As CrcZ binds to the distal site of Hfq it is conceivable that Hfq might even still be able to bind sRNAs such as PrfF at the proximal site, and thus to protect them from degradation. In any case it will be interesting to study whether sequestration of Hfq by CrcZ indirectly affects other Hfq-mediated processes in *P. aeruginosa*.

### The elusive function of Crc in CCR of *Pseudomonas*

We have recently shown that the Crc protein is devoid of RNA binding activity [10], and that the previously observed RNA binding activity of Crc could be attributed to Hfq [11]. However, Crc contributes to Hfq-mediated repression during CCR (Figure 1). In addition, Crc was shown to impact on biofilm formation [54,55], virulence and antibiotic susceptibility [52], traits which are also affected by Hfq [31,33]. This raises the question how Crc impacts on Hfq function. Crc appears not to interfere with *hfp* expression. The translation of a *hfp*-lacZ reporter gene, whose transcription is driven by the authentic *hfp* promoter, was indistinguishable in PAO1 and in the isogenic PAO1Δcrc strain (Figure S10A). In addition, the levels of Hfq, as determined by quantitative western-blot analysis, were not significantly altered in PAO1Δcrc when compared with the wild-type strain (Figure S10B). Conversely, Hfq seems not to affect the cellular concentration of Crc (Figure S10B). Thus, Crc seems not to impact on the Hfq levels, and *vice versa* Hfq seems not to affect that of Crc.

RelA was recently shown to enhance multimerization of *E. coli* Hfq, and thereby to stimulate binding to sRNAs [56]. We therefore considered the possibility that Crc might interact with Hfq to increase the specificity of Hfq for A-rich sequences. However, as revealed by EMSA assays, the presence of Crc did not increase the affinity of PAO1 Hfq for *amiE*-134–20 RNA (E. Sonnleitner, unpublished), making it less likely that Crc acts similar to RelA. Nevertheless, we are currently exploring the possibility whether Crc is associated with Hfq in vivo.

### Materials and Methods

#### Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table S2. Unless indicated otherwise, the cultures were grown at 37°C in BSM minimal medium supplemented with 20 mM succinate. The strains PAO1*hfp-Darc* and PAO1*hfp-Darc crcZ* were constructed by homologous recombination. Briefly, plasmid pME9672 and plasmid pME9673, respectively, were mobilized into strain PAO1*hfp* with the aid of *E. coli* strain HB101(pRK2013), and then chromosomally integrated through selection for tetracycline resistance. Excision of the vector by a second crossover event was achieved by enrichment for tetracycline-sensitive cells [57]. If required, *E. coli* and PAO1 were grown in the presence of 100 μg ml$^{-1}$ ampicillin, 23 μg ml$^{-1}$ tetracycline or 25 μg ml$^{-1}$ kanamycin and 50 μg ml$^{-1}$ gentamicin, 100 μg ml$^{-1}$ tetracycline or 250 μg ml$^{-1}$ carbenicillin, respectively. Details on the construction of plasmids used in this study are provided in Text S1.

#### β-Galactosidase assays

The β-galactosidase activities were determined as described by Miller [58]. The cells were permeabilized with 5% toluene. The β-galactosidase units in the different experiments were derived from 1 ml aliquots of the respective cultures withdrawn; the cells were harvested by centrifugation, resuspended and boiled in protein sample buffer. Equal amounts of total protein were separated on 12% SDS-polyacrylamide gels and then electroblotted to a nitrocellulose membrane. The blots were blocked with 5% dry milk in TBS buffer, and then probed with rabbit anti-DYKDDDDK polyclonal antibody (Roth). The antibody-antigen complexes were visualized with alkaline-phosphatase conjugated secondary antibodies (Sigma) using the chromogenic substrates nitro blue tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP).

#### Protein purification

Hfq protein, Hfq$_{V22D}$ and Hfq$_{K56A}$ protein were produced in the *hfp* deficient *E. coli* strain AM111F, harboring plasmid pRUES2514 for *hfp* expression. Hfq$_{V22D}$ and Hfq$_{K56A}$ were overproduced in the absence of the *hfp* gene, whose transcription is driven by the authentic *hfp* promoter, was indistinguishable in PAO1 and in the isogenic PAO1Δcrc strain (Figure S10A). In addition, the levels of Hfq, as determined by quantitative western-blot analysis, were not significantly altered in PAO1Δcrc when compared with the wild-type strain (Figure S10B). Conversely, Hfq seems not to affect the cellular concentration of Crc (Figure S10B). Thus, Crc seems not to impact on the Hfq levels, and *vice versa* Hfq seems not to affect that of Crc.

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#### Western-blot analyses

The protein levels of Hfq and Crc fused to C-terminal Flag-tags (DYKDDDDK) were determined in the respective strains and under the growth conditions as specified in the legends to the Figures. 1 ml aliquots of the respective cultures were withdrawn; the cells were harvested by centrifugation, resuspended and boiled in protein sample buffer. Equal amounts of total protein were separated on 12% SDS-polyacrylamide gels and then electroblotted to a nitrocellulose membrane. The blots were blocked with 5% dry milk in TBS buffer, and then probed with rabbit anti-DYKDDDDK polyclonal antibody (Roth). The antibody-antigen complexes were visualized with alkaline-phosphatase conjugated secondary antibodies (Sigma) using the chromogenic substrates nitro blue tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP).

#### In vitro transcription

For *in vitro* transcription of *amiE* (1172 nt), CrcZ (426 nt) and RsmZ (141 nt) RNAs the AmpliScribe T7-Flash Transcription Kit (Epigenic Biotechnologies) was used according to the manufacturer’s instructions. First, PCR fragments were generated with the primer pairs (see Table S3) A5/AT7 (*amiE*), E6/C6 (crcZ) and W26/X26 (rsmZ), whereby the forward primers contained T7 promoter sequences. Primer AT7 encoded in addition a Flag-tag sequence. For the filter binding and gel mobility shift assays truncated versions of *amiE*-134–20 (first 154 nt) and crcZ$_{513}$ (first
Enzymatic probing of RNA

Five pmol of in vitro transcribed full length amiE RNA was incubated in RT-buffer (50 mM Tris pH 8.3, 60 mM NaCl, 6 mM Mg-aceate, 10 mM DTT) at 37°C for 30 min in a 10 µl reaction with 0, 5 and 20 pmol of purified Hfq protein. Then 2 U RNase T1 (1 µl), 10 pg RNase A (1 µl) or 1 µl of RNase free H2O was added and incubated for additional 10 min followed by phenol/chloroform extraction and precipitation. 200 fmol RNase treated or untreated RNAs were further used for the primer extension reaction with AMV reverse transcriptase (Promega), which was primed with the 5'-[³²P]-labeled C78 oligonucleotide to test for protection by Hfq of the proximal part of amiE mRNA. For sequencing amiE RNA and dNTPs (Fermentas) were used in primer extension reaction(s).

Toeprint analysis

The amiE_{134-476} RNA used for toeprinting was obtained as described above. The [³²P]-5'-end labeled oligonucleotide Q99 was annealed to amiE mRNA (+57 to +76 with regard to the A (+1) of the start codon) and used to prime cDNA synthesis by MMuLV reverse transcriptase (Promega). The toeprinting assay was carried out with purified E. coli 30S ribosomal subunits and E. coli initiator-tRNA (tRNA^{Met}) as described by Hartz et al. [37]. The mRNA (0.05 pmol) was pre-incubated at 37°C for 10 min with or without 4 pmol 30S subunits and 16 pmol tRNA^{Met} before reverse transcriptase was added. To test whether Hfq interferes with translation initiation, 0.05 pmol amiE_{134-476} mRNA was pre-incubated at 37°C for 10 min with 4 pmol 30S subunits, 16 pmol tRNA^{Met} and Hfq-hexamer (1 or 2 pmol, respectively) before reverse transcriptase reaction was performed. To test whether CrcZ can abrogate the Hfq mediated repression of amiE translation initiation 0.05 pmol amiE_{134-476} mRNA was pre-incubated at 37°C for 10 min with 4 pmol 30S subunits, 16 pmol tRNA^{Met} and equimolar amounts of Hfq-hexamer and CrcZ (1 or 2 pmol, respectively) before reverse transcriptase was added.

Electro mobility shift assays

The amiE_{134-420} and crcZ_{151} RNAs (see above) were dephosphorylated with FastAP thermo sensitive alkaline phosphatase (Thermo Scientific) and subsequently 5'-end labeled using [γ-³²P]-ATP (Hartmann Analytic) and polynucleotide kinase (Thermo Scientific). The labeled RNAs were gel-purified and dissolved in diethylpyrocarbonate-treated water. Labeled RNA (10 nM) was incubated with increasing amounts of purified Hfq, the HfqK235D or HfqK566A mutant proteins in 10 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 60 mM NaCl, 10 mM NaH₂PO₄, 10 mM DTT, and 25 ng tRNA in a total volume of 10 µl. Unlabeled RNA was used as competitor as stated in the legend to Figure 5A. The reaction mixtures were incubated at 37°C for 30 min to allow protein-RNA complex formation. The samples were mixed with 4 µl loading dye (25% glycerol, 0.2 mg/l xylenol and bromphenol blue) immediately before loading and separated on 4% polyacrylamide gels using Tris-borate buffer. The radioactively labeled bands were visualized with a PhosphorImager (Molecular Dynamics) and quantified with ImageQuant software 5.2.

In vitro translation

In vitro translation was performed with the PURExpress in vitro protein synthesis kit (New England BioLabs). 5 pmol in vitro transcribed amiE<sub>Flag</sub> mRNA was used in a 12.5 µl reaction. Increasing amounts of purified Hfq protein (as specified in legends of Figures 5B and S4) were added. For competition CrcZ (5 and 10 pmol) or RsmZ (10 pmol) RNA were added. After 1 h of incubation at 37°C, 5 µl of the reaction was mixed with 5 µl protein loading buffer and a western-blots was performed using anti-Flag antibodies as described above.

Esterase assay

Esterase activity was assayed as described previously [60]. Briefly, the cells were harvested by centrifugation and washed in 100 mM potassium phosphate buffer pH 7.2. The substrate (25 µl p-nitrophenyl-caproate dissolved in 5 ml ethanol) was added to 100 ml potassium phosphate buffer (100 mM; pH 7.2) containing MgSO₄ to a final concentration of 10 mM. 1 ml of the test solution and 50 µl of cells were used to determine esterase activity by monitoring the change in absorbance at 410 nM min⁻¹, which was normalized to the optical density (OD<sub>600</sub>) of the culture.

Northern-blot analyses

Total RNA of the respective strains as specified in the legends to the Figures was purified using hot phenol. The steady state levels of CrcZ and 5S rRNA (loading control) were determined by Northern-blotting using 4 µg of total RNA. The RNA samples were denatured for 5 min at 65°C in loading buffer containing 50% formamide, separated on a 8% polyacrylamide/8 M urea gel, and then transferred to a nylon membrane by electroblotting. The RNAs were cross-linked to the membrane by exposure to UV light. The membranes were hybridized with gene-specific ³²P-end-labelled oligonucleotides (CrcZ; K3; 5S rRNA: I26; Table S3). The hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

Supporting Information

Figure S1  CCR represses amiE at the post-transcriptional level. (A) The strains were grown to an OD<sub>600</sub> of 2.0 in BSM medium supplemented with 40 mM succinate and 40 mM acetamide (blue bar; CCR) or with 40 mM mannitol and 40 mM acetamide (grey bar; no CCR). Acetamide was added to induce transcription of the amiE-lac<sub>Z</sub> fusion. Then, cells were harvested and the activities were determined. The bars depict the β-galactosidase activities. The bars depict the β-galactosidase values conferred by the transcriptional amiE- lac<sub>Z</sub> fusion encoded by plasmid pTCamiE (left panel) and by the translational amiE-lac<sub>Z</sub> fusion encoded by plasmid pME9655 (right panel) in strains PAO1(pTCamiE) and PAO1(pME9655), respectively. (B) The strains were grown to an OD<sub>600</sub> of 2.0 in BSM medium supplemented with 40 mM succinate and 40 mM acetamide. Then, the cells were harvested and the β-galactosidase activities were determined. The bars depict the β-galactosidase values conferred by the transcriptional amiE- lac<sub>Z</sub> fusion encoded by plasmid pTCamiE in strains PAO1 (blue bar), PAO1Δcrc (green bar), PAO1Δhfg- (red bar) and PAO1Δhfg-Δcrc (purple bar), respectively. (C) Hfq does not affect lac<sub>Z</sub> expression. The strains were grown to an OD<sub>600</sub> of 2.0 in BSM medium supplemented with 40 mM succinate and 1 mM IPTG. Then, the cells were harvested and the β-galactosidase activities were determined. The bars depict the β-galactosidase values conferred by the lac<sub>Z</sub> gene encoded by plasmid pME3856 in strains PAO1 (blue bar), PAO1Δcrc (green bar), PAO1Δhfg- (red bar) and PAO1Δhfg-Δcrc (green bar).
**Figure S2** Hfq binds to amiE mRNA and CrcZ RNA in vivo during mimicked CCR. Strains PAO1 hfq-::pMBM67HE and PAO1 hfq-::pMBM67AHE (control) were grown under conditions of mimicked CCR in BSM medium supplemented with 40 mM succinate, 40 mM acetamide (transcriptional induction of amiE) and 1 mM IPTG (transcriptional induction of hfq). Lanes 1 and 2, RT-PCR with amiE, CrcZ and RsmZ specific oligonucleotides performed with RNA obtained from the supernatant after co-immunoprecipitation (S) and after mock co-immunoprecipitation (CoIP) in the absence of Hfq in lysates of strain PAO1 hfq-::pMBM67HE. Lanes 3 and 4, RT-PCR with amiE, CrcZ and RsmZ specific oligonucleotides performed with RNA obtained from the supernatant after co-immunoprecipitation (S) and after mock co-immunoprecipitation (CoIP) in the absence of Hfq in strain PAO1 hfq-::pMBM67HE. Lane 5, chromosomal DNA of PAO1 served as a positive control. Lane 6, “RT-PCR” reaction without reverse transcriptase (negative control; -RT) with RNA obtained after CoIP with Hfq-specific antibodies, i.e., as used in the reactions shown in lane 2.

**Figure S3** Filter binding assay of amiE-134–420 with increasing amounts of Hfq-hexamer. The percentage of bound RNA is plotted against the concentration of Hfq-hexamer. The experiment was performed in duplicate.

**Figure S4** Repression of amiE Flag mRNA translation in the presence of Hfq. Lane 1, in vivo translation of amiE Flag mRNA. Lanes 2–7, inhibition of amiE Flag mRNA translation in the presence of increasing amounts of Hfq. The molar ratios of Hfq hexamer (Hfq6) to amiE Flag mRNA are denoted on top.

**Figure S5** (A) EMSA with 10 nM radioactively labeled amiE-134–420 RNA in the presence of increasing amounts of Hfq, HfqY25D and HfqK56A. Lane 1, labeled amiE-134–420 RNA without addition of protein. Lanes 2–4, lanes 5–7 and lanes 8–10, Hfq, HfqY25D and HfqK56A were added in 5, 10 and 20-fold molar excess over amiE-134–420 RNA, respectively. (B) Repression of amiE translation by PAO1 Hfq in E. coli. The strain JW4130(pME9650) concomitantly harboring the control plasmid pME4510 (red bar), plasmid pME4510/hfqrac (blue bar), plasmid pME4510/hfqrac::gfp (orange bar) and pME4510/amiE (purple bar), respectively, was grown in LB broth to an OD600 of 2.0. Then, the β-galactosidase activity conferred by the translational amiE::lacZ fusion encoded by plasmid pME9650 was determined. Inset: the levels of the PAO1 Hfq, PAO1 HfqY25D and PAO1 HfqK56A proteins were determined by quantitative western-blot analysis using anti-Flag antibodies. The error bars represent standard deviations from three independent experiments.

**Figure S6** Hfq binds to CrcZ with its distal face. (A) Schematic representation of CrcZ RNA. Potential Hfq binding sites are depicted in red. (B) EMSA with 10 nM radioactively labeled CrcZ151 RNA in the presence of increasing amounts of Hfq, HfqY25D and HfqK56A. Lane 1, labeled CrcZ151 RNA without addition of protein. Lanes 2 and 3, lanes 4 and 5 and lanes 6 and 7, Hfq, HfqY25D and HfqK56A were added in 5 and 20-fold molar excess over CrcZ151 RNA, respectively.

**Figure S7** Hfq is required for CrcZ function. The strains were grown to an OD600 of 0.5 in BSM medium supplemented with 40 mM succinate and 40 mM acetamide. Then IPTG was added (1 mM final concentration). At an OD600 of 2.0 the cells were harvested and the β-galactosidase activities were determined. The bars depict the β-galactosidase values conferred by the translational amiE::lacZ fusion encoded by plasmid pME9655 in strain PAO1 harboring either the control plasmid pMBM67HE (blue bar) or the crcZ encoding plasmid pMBMErcZ (pink bar) (A) and in strain PAO1 hfq-::pMBM67HE (red bar) or the crcZ encoding plasmid pMBM67HE (pink bar) (B), respectively. The error bars represent standard deviations from three independent experiments. The CrcZ levels (top panel) were determined by Northern-blot analysis. 18S rRNA served as a loading control.

**Figure S8** CCR results in destabilization of amiE mRNA. PAO1 was grown in BSM medium supplemented with 40 mM acetamide (No CCR) or supplemented with 40 mM acetamide and 40 mM succinate (CCR). PAO1 hfq-::pMBM67HE was grown in BSM medium supplemented with 40 mM acetamide and 40 mM succinate (CCR). At an OD600 of 1.0, rifampicin was added to a final concentration of 100 μg/ml and samples were withdrawn for total RNA extraction at the times indicated. (A) The remaining levels of amiE and 16S rRNA (control) were determined by RT-PCR with oligonucleotides specific for either RNA as described in Text S1. The result from one representative experiment is shown. (B) The amounts of amiE mRNA during CCR in PAO1 (blue squares) and in PAO1 hfq-::pMBM67HE (red triangles), respectively, as well as in PAO1 in the absence of CCR (green diamonds) were normalized to that of 16S rRNA at different times after addition of rifampicin. The results are derived from three independent experiments. Error bars represent standard deviations. The half-life of amiE mRNA was determined with 6.8+/-0.4 min in PAO1 and with 10.1+/-0.5 min in PAO1 hfq-::pMBM67HE during CCR. In the absence of CCR the half-life of amiE mRNA was determined with 11.4+/-0.8 min.

**Figure S9** Determination of the cellular Hfq concentration. PAO1 was grown in BSM medium supplemented with 40 mM succinate to an OD600 of 2.0, = 3.2+0.3 109 CFU/ml). The Hfq concentration was determined in triplicate samples of PAO1 cell lysates corresponding to 50 μl of culture (lanes 2–4) using quantitative western-blotting with Hfq specific antibodies. Lane 1, marker protein. Lanes 5–7, 0.1, 0.4 and 0.6 pmol of purified Hfq protein were loaded, respectively. The Hfq concentration per cell was determined as described in Text S1.

**Figure S10** Crc does not affect hfq expression. (A) Determination of the β-galactosidase activity conferred by the translational hfq::lacZ fusion encoded by plasmid pTLhfq during growth in strain PAO1 (blue squares) and in strain PAO1Δcrc (green circles). The strains were grown in BSM medium supplemented with 40 mM succinate. The error bars represent standard deviations from three independent experiments. (B) Levels of Hfq and Crc in PAO1 (wt), PAO1Δcrc (Δcrc) and PAO1Δhfq–ΔΔhfq grown to an OD600 of 2.0 in BSM medium supplemented with 40 mM succinate. Immunodetection of Hfq, Crc and of ribosomal protein S2 (loading control) was performed as described in Text S1.
Table S1: Catabolic and transport genes that are up-regulated in the absence of Hfq.

Table S2: Strains and plasmids used in this study.

Table S3: Oligonucleotides used in this study.

Text S1: Supporting materials and methods and references.

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Author Contributions
Conceived and designed the experiments: ES UB. Performed the experiments: ES. Analyzed the data: ES UB. Contributed reagents/materials/analysis tools: ES UB. Wrote the paper: ES UB.
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