Interplay between the catabolite repression control protein Crc, Hfq and RNA in Hfq-dependent translational regulation in Pseudomonas aeruginosa

Elisabeth Sonnleitner1,*, Alexander Wulf2, Sébastien Campagne3, Xue-Yuan Pei4, Michael T. Wolfinger5,6, Giada Forlani1, Konstantin Prindl1, Laetitia Abdou7, Armin Resch1, Frederic H.-T. Allain3, Ben F. Luisi4, Henning Urlaub2,8 and Udo Bläsi1,*

1Department of Microbiology, Immunobiology and Genetics, Max F. Perutz Laboratories, University of Vienna, Vienna Biocenter, Dr. Bohrgasse 9, 1030 Vienna, Austria, 2Biophysical Mass Spectrometry Group, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany, 3Institute of Molecular Biology and Biophysics, ETH Zürich, 8093 Zürich, Switzerland, 4Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, UK, 5Institute of Theoretical Chemistry, University of Vienna, 1090 Vienna, Austria, 6Center for Anatomy and Cell Biology, Medical University of Vienna, 1090 Vienna, Austria, 7Department of Fundamental Microbiology, University of Lausanne, 1015 Lausanne, Switzerland and 8Bioanalytics, Institute for Clinical Chemistry, University Medical Center Göttingen, 37075 Göttingen, Germany

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ABSTRACT

In Pseudomonas aeruginosa the RNA chaperone Hfq and the catabolite repression control protein (Crc) act as post-transcriptional regulators during carbon catabolite repression (CCR). In this regard Crc is required for full-fledged Hfq-mediated translational repression of catabolic genes. RNAseq based transcriptome analyses revealed a significant overlap between the Crc and Hfq regulons, which in conjunction with genetic data supported a concerted action of both proteins. Biochemical and biophysical approaches further suggest that Crc and Hfq form an assembly in the presence of RNAs containing A-rich motifs, and that Crc interacts with both, Hfq and RNA. Through these interactions, Crc enhances the stability of Hfq/Crc/RNA complexes, which can explain its facilitating role in Hfq-mediated translational repression. Hence, these studies revealed for the first time insights into how an interacting protein can modulate Hfq function. Moreover, Crc is shown to interfere with binding of a regulatory RNA to Hfq, which bears implications for riboregulation. These results are discussed in terms of a working model, wherein Crc prioritizes the function of Hfq toward utilization of favored carbon sources.

INTRODUCTION

The opportunistic pathogen Pseudomonas aeruginosa can utilize numerous carbon sources, which permits growth under diverse environmental conditions. The uptake and assimilation of carbon is controlled by carbon catabolite repression (CCR), a process that ensures that the utilization of less preferred carbon sources (e.g. mannitol or acetamide) is impeded until the preferred one (e.g. succinate) is consumed (1). CCR not only impacts on metabolic regulation, but is also linked to complex behavior including biofilm formation, quorum sensing, virulence and antibiotic susceptibility (2–5).

In contrast to CCR in Enterobacteriaceae and Firmicutes (6), in Pseudomonas CCR operates at the post-transcriptional level and employs the RNA chaperone Hfq, the catabolite repression control protein Crc and the regulatory RNA CrCZ (1,7). Recent studies provided evidence that Hfq acts as a translational repressor during CCR in P. aeruginosa (7). During growth on succinate several catabolic genes of P. aeruginosa were shown to be translationally silenced by Hfq, which can bind with its distal face to A-rich sequences within or adjacent to ribosome binding sites. Upon relief of CCR, e.g. after exhaustion of succinate and continued growth on mannitol, the level of the Hfq-binding RNA CrCZ increases (8), leading to sequestration of Hfq. This in turn abrogates Hfq-mediated translational repression of the respective catabolic genes (7).

The catabolite repression control protein Crc has been implicated in multicellular behavior and biofilm formation.

8To whom correspondence should be addressed. Tel: +43 1 4277 54606; Email: elisabeth.sonnleitner@univie.ac.at

Correspondence may also be addressed to Udo Bläsi. Tel: +43 1 4277 54609; Email: udo.blaesi@univie.ac.at

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A crc mutant was impaired in swimming, swarming and twitching motility, and showed defects in initial biofilm development (2,4,9). Some of these phenotypes were also observed with a PAO1 hfq− mutant (10), indicating early on that Hfq and Crc may act together. In fact, recent studies suggested that both, Hfq and Crc, mediate post-transcriptional regulation during CCR as full-fledged repression of catabolic genes by Hfq required Crc (7,11).

In Enterobacteriaceae, Hfq is pivotal for riboregulation, which results on the one hand from binding to and protection of small regulatory RNAs (sRNA) from nucleolytic decay, and on the other hand from accelerating the annealing between sRNAs and their target mRNAs (reviewed in 12). The P. aeruginosa Hfq, which lacks the C-terminal extension present in enterobacteriaceal counterparts, has likewise been shown to accelerate annealing of two RNA substrates (13,14) as well as being required for riboregulation of antR mRNA by the sRNAs PrfF1-2 (14) and of algC mRNA by the sRNA ErsA (15). Interactome studies performed with Escherichia coli Hfq (16) and P. aeruginosa Hfq (17) revealed a large number of putative interacting proteins with functions in transcription, translation and mRNA decay. Several candidate proteins including RNA polymerase, ribosomal protein S1 (18), RNase E (19), polyA-polymerase and polynucleotide-phosphorylase (20) have been found to associate with E. coli Hfq. However, several follow up studies did not reveal a direct physical interaction between Hfq and these candidate proteins (21–23). Most likely, these complexes are RNA-mediated and result from the spatial association of the transcriptional, translational and RNA decay machineries. Similarly, in P. aeruginosa components of the degradosome were shown to co-purify with Hfq (17). Interestingly, the P. aeruginosa Crc protein co-purified as well with tagged Hfq protein (17). Moreover, pull-down assays indicated that P. putida Hfq and Crc form a co-complex in the presence of CrcZ RNA (11).

Here, using an E. coli two-hybrid system and co-immunoprecipitation (co-IP), we first show that Hfq and Crc associate in vivo. Biochemical and biophysical experiments extend findings from recent studies (11), and show that the Hfq/Crc interaction requires RNA bound to the distal side of Hfq. Crc bound neither to Hfq nor to RNA alone (24). However, as revealed by RNA cross-linking, Crc interacts with both Hfq and RNA in Hfq/Crc/RNA complexes. The multivalency inherent to Crc apparently increases the stability of these complexes when compared with Hfq/RNA complexes, whereas it appears not to affect selectivity. Moreover, our data indicate that Crc interferes with the binding of a sRNA to Hfq. The implications of the latter finding for riboregulation are discussed.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids and growth conditions**

The strains and plasmids used in this study are listed in Supplementary Table S1. Details on the construction of plasmids and strains are provided in Supplementary Text S1. Unless indicated otherwise, the cultures were grown at 37°C in BSM medium (30.8 mM K2HPO4, 19.3 mM KH2PO4, 15 mM (NH4)2SO4, 1 mM MgCl2 and 2 µM FeSO4) supplemented with the indicated carbon sources. If required, E. coli was grown in the presence of 100 µg ml−1 ampicillin, 25 µg ml−1 tetracycline and 25 µg ml−1 kanamycin, respectively and P. aeruginosa was grown in the presence of 50 µg ml−1 gentamicin, 100 µg ml−1 tetracycline and 250 µg ml−1 carbenicillin, respectively.

**RNAseq library construction and sequence analysis**

Total RNA was prepared from two biological replicates of strains PAO1, PAO1Δcrc and PAO1Δhfq−, respectively, after growth in BSM complex medium (BSM medium containing 40 mM succinate, 5 mM of acetate, glucose, mannitol, acetamide, histidine, tryptophan, phenylalanine, leucine, isoleucine, glutamate, arginine, valine and lysine, 0.25 mM anthranilate and 0.25% glycerol) to an OD600 of 1.5. Then, 10 ml samples were withdrawn and total RNA was extracted using the hot phenol method (25), contaminating DNA was removed by DNase (Roche) treatment followed by phenol-chloroform (pH 5.5) extraction and ethanol precipitation. To remove ribosomal RNAs, the Ribo-Zero® Magnetic Kit for Gram-negative bacteria (Epicentre) was used according to the manufacturer’s instructions. Libraries were constructed using NEBNext® Ultra™ Directional RNA Library Prep Kit from Illumina. 100 base pair single end sequence reads were generated using the Illumina HiSeq 2000 platform at the Vienna BioCenter Core Facility (http://www.csf.ac.at). Sequencing adapter removal was performed with cutadapt (26). Mapping of the samples against the PAO1 reference genome (NCBI accession number NC_002516.2) was performed with Segemehl (27) with default parameters. Reads mapping to regions annotated as either rRNA or tRNA were discarded from all data and ignored for all follow up analysis steps. The mapped sequencing data were prepared for visualization using the ViennaNGS tool box, and visualized with the UCSC Genome Browser (28). Reads per gene were counted using BEDTools (29) and the Refseq annotation of P. aeruginosa (NC_002516.2). Differential gene expression analysis was performed with DESeq (30). All RNAs with a fold-change greater than 5 and a multiple testing adjusted P-value below 0.05 were considered to be differentially abundant. The raw sequencing data were deposited in the European nucleotide archive (ENA) as a study under the accession number PR-JEB22802.

**Bacterial adenylate cyclase-based two-hybrid system (BACTH)**

Plasmids encoding C- and N-terminal fusion-proteins of Hfq and Crc with the catalytic domains T25 and T18, respectively, of Bordetella pertussis adenylate cyclase were constructed (Supplementary Text S1) and co-transformed into the cya deficient E. coli strain BTH101. BACTH was performed as previously described (31). Briefly, the interaction between two hybrid proteins was quantified by determining the β-galactosidase activity, which in turn depends on the intracellular cAMP levels. Escherichia coli strain BTH101, harboring the respective plasmids, was grown in Luria broth (32) to an OD600 of 0.7. Then, 1 mM IPTG was added, and 1 h thereafter the cells were harvested and the β-galactosidase activity was determined.
β-galactosidase assays

The β-galactosidase activities were determined as described (32). The cells were permeabilized with 5% toluene. Unless indicated otherwise, the β-galactosidase units in the different experiments were derived from three independent experiments and are shown as mean. The error bars in the different Figures represent standard deviations.

In vivo and in vitro co-immunoprecipitation studies

For in vivo co-IP, PAO1, PAO1Δhfq and PAO1Δcrc were grown in BSM complex medium as described above (40 ml of culture) and harvested at an OD600 of 1.5. The cells were first washed in lysis buffer (20 mM Tris pH 8.0, 150 mM KCl, 1 mM MgCl2, 1 mM DTT, 0.05% Triton X-100) and then snap frozen in liquid nitrogen. The cells were lysed by sonication (six times for 10 s on ice) in 800 μl lysis buffer in the presence of 200 U RiboLock® RNAse inhibitor (Fermentas). Cell debris were removed by centrifugation and anti-Hfq antibodies (Pineda) were added to 60 μl supernatant and incubated for 2 h at 4°C on a rotating wheel. Then, 5 μl Dynabeads® Protein G beads (Novex) were added and the incubation was continued for 1 h. The beads were washed three times with lysis buffer and finally collected in 25 μl of SDS loading dye. 5 μl were used for further analysis by western-blotting.

For in vitro co-IP studies, 40 pmol of Hfq-hexamer (HfqH) and 120 pmol of Crc protein with or without 40 pmol RNA were incubated for 30 min at 37°C in 200 μl ES-buffer (10 mM Tris pH 8.0, 10 mM KCl, 40 mM NaCl and 1 mM MgCl2) in the presence of 0.05% Triton X-100. Then, 10 μl of rabbit anti-Hfq antibodies (Pineda) were added and the incubation was continued for 30 min at 4°C on a rotating wheel. Thereafter, 5 μl Dynabeads® Protein G beads (Novex) were added for 30 min. The beads were washed three times with ES-buffer and finally collected in 50 μl of SDS loading dye. 5 μl were used for further analyses by western-blotting.

Western-blot analyses

Equal amounts of co-immunoprecipitated or total proteins were separated on 12% SDS-polyacrylamide gels, and then electro-blotted onto a nitrocellulose membrane. The blots were blocked with 5% dry milk in TBS buffer, and probed with rabbit anti-Hfq (Pineda), or rabbit anti-Crc (Pineda) antibodies. Immunodetection of ribosomal protein S1 served as a loading control. 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).

NMR

The Crc protein was produced in Escherichia coli BL21(DE3)(pET26bII-Crc). Crc was deuterated by growing the cells in M9 minimal medium containing 100% D2O (~80% of the protons were replaced by deuterium), in the presence of 15N-labeled NH4Cl (1g/l) or in the presence of alpha-ketoisovaleric acid (13C5, 98%; 3-D1, 98%) to ensure specific 13C-labeling of leucine and valine methyl groups. The cells were grown at 37°C to an OD600 of 0.6. Then IPTG was added to a final concentration of 0.5 mM for 18 h at 15°C. The protein was then purified as described (33). All NMR measurements were performed at 313 K on an Avance III 900 MHz spectrometer. For the NMR titration of 15N-labeled Crc by Hfq, the 15N-1H fingerprint of Crc was monitored upon addition of Hfq by recording 2D 15N-1H BEST-TROSY HSQC. For the titration of 13C-labeled Crc, the signals for Crc were monitored upon addition of the Hfq/RNA complex by recording 2D 13C-1H HMBC spectra.

Microscale thermophoresis (MST)

MST is based on the directed movement of molecules along temperature gradients. Any change of the hydration shell of biomolecules due to changes in their structure/conformation results in a relative change of the movement along the temperature gradient, which can be used to determine binding affinities (34). 20 μM of purified Crc and Hfq proteins were labeled with Monolith NT™ Protein Labeling Kit RED-NHS according to the manufacturer’s instructions (Nano Temper). To study Hfq-rRNA interactions, PrR2F2 sRNA was labeled at the 3′-end using T4 RNA Ligase (NEB) and pCy-Cy5 (Jena Bioscience) according to the manufacturer’s instructions. For determination of protein-protein or protein-RNA interactions 40 nM PrR2F2-Cy5, 30 nM labeled Hfq and—due to a weaker labeling efficiency—200 nM labeled Crc protein, respectively, were used in the presence of increasing amounts of ligands (either Hfq, Crc, amiEARN RNA-oligonucleotide (5′-AAAAAUAACAAAGG-3′; purchased from Sigma) or combinations thereof, as indicated in the Results. The ligands were dissolved in ES-buffer (10 mM Tris pH 8.0, 10 mM KCl, 40 mM NaCl and 1 mM MgCl2) in the presence of 0.05% Triton X-100. After 2 min incubation at room temperature, the samples were loaded onto MST Premium coated capillaries (Nano Temper) and measured in a Monolith NT.115 instrument at the Vienna BioCenter Core Facility (http://www.csf.ac.at). The MST measurements were performed in duplicate. If not indicated otherwise, the following parameters were used: LED Power 90%, MST Power 60%. Data analysis was performed with NTAffinityAnalysis v2.0.2 for thermophoresis and T-jump analysis 0 and 5 s after the pulse. For determination of the Kd-values the Hill Model was used that is included in the NTAffinityAnalysis software.

RNA-Protein cross-linking

To reconstitute the RNA-protein complex, 1 nmol Hfq and 1 nmol amiEARN RNA were incubated with 3 nmol Crc in 200 μl ES-buffer for 30 min at 37°C. Substituting Crc with additional ES-buffer served as a negative control. Samples were split evenly into two aliquots, one of which was UV-irradiated at 254 nm, while the other served as a non-irradiated control. Further sample processing was performed as described in detail in Sharma et al. (35).
Protein-Protein cross-linking

Freshly prepared Hfq/Crc amiE\textsubscript{6ARN} complexes were used for chemical cross-linking with the amine-reactive, water-soluble, homobifunctional protein cross-linker bis(sulfosuccinimidyl) suberate (BS3; Thermo Fisher Scientific). A 263-times molar excess of the cross-linker over the Hfq/Crc amiE\textsubscript{6ARN} complex was used; 480 pmol of the Hfq/Crc amiE\textsubscript{6ARN} complex was incubated with 126 nmol of BS3 and incubated on ice for 2 h. After the incubation period, the reaction was quenched by addition of 1 \( \mu \)l of 200 mM Tris–HCl (pH 8.0). The cross-linked samples were then subjected to SDS-PAGE on a 4–12% Bis–Tris gel (Invitrogen) with MOPS as running buffer. The gel was stained using Coomassie brilliant blue G250 and de-stained in water. The identified bands, corresponding to cross-linked protein-protein conjugates, were excised and subjected to in-gel trypsin or trypsin/Lys-C digestion in a 1:5 ratio of total protein to the enzyme mix (Trypsin LysC Mix; Promega). The proteolytic peptides were extracted and resuspended in 2% acetonitrile (ACN) and 0.05% trifluoro acetic acid (TFA) in a final volume of 14 \( \mu \)l and subjected to liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis.

Mass spectrometry (MS/MS) and MS data analysis

The samples were loaded onto a self-packed C18 column, mounted on a Dionex Ultimate 300 UHPLC (Thermo Scientific): 3 \( \mu \)m pore size, 75 \( \mu \)m in diameter, 30 cm in length (Reprosil-Pur\textsuperscript{12} 120C18-AQ, Dr Maisch GmbH). The peptides were separated by reverse-phase chromatography on a 58 min multi-step gradient with a flow rate of 0.3–0.4 \( \mu \)l/min before entering the mass spectrometer (QExactive HF, Thermo Scientific). MS1 spectra were recorded in profile mode with a resolution of 120k, whereas MS2 spectra were recorded in centroid mode with a resolution of 30k. The isolation window was set to 1.6 m/z and the dynamic exclusion was set to 9 s. The raw data of RNA–protein heteroconjugates were analyzed and manually validated with the OpenMS pipeline RNPsxl (36). The raw data of protein-protein crosslinked spectra were analysed and validated using pLink (37). Structural visualization of results was performed with Chimera 1.1.2. (38).

Electrophoretic mobility shift assays (EMSA)

For \textit{in vitro} transcription of CrcZ (426 nt) and PrrF2 (107 nt) RNA the AmpliScribe T7-Flash Transcription Kit (Epigene Technologies) was used according to the manufacturer’s instructions. First, PCR fragments were generated with the primer pairs E6 (5′-TCT AGA CGT AAT ACG ACT CAC TAT AGG CAC AAC AAT AAC AAG C-3′) and C6 (5′-ATG CGG ATC CGA AAT GGT GTA AGG CGG AGG-3′) (crcZ) and W77 (5′-TTT TTT TTT TCT AGA CGT AAT ACG ACT CAC TAT AGG CAC TCG GAG GCC C-3′) and X77 (5′-CAA AAA AAG ACC CGG CAA AG-3′) (prrF2) and chromosomal DNA of P. aeruginosa PAO1. The forward primers contain a T7 promoter sequence (underlined).

To determine whether Crc affects Hfq-RNA complex formation, the RNA oligonucleotide amiE\textsubscript{6ARN} was 5′-end labeled with [\( \gamma \)-\( ^{32} \)P]-ATP (Hartmann Analytic) and polynucleotide kinase (Thermo Scientific), and the labeled RNA was extracted using phenol-chloroform followed by ethanol precipitation. 10 nM labeled RNA was incubated in ES buffer (10 mM Tris pH 8.0, 10 mM KCl, 40 mM NaCl and 1 mM MgCl\textsubscript{2}) with increasing amounts of purified Hfq protein as specified in the legend to Figure 4A in the presence or absence of 480 nM Crc (3-fold molar excess over the highest concentration of Hfq) and 25 ng tRNA in a total volume of 10 \( \mu \)l.

To assess the stability of RNA/Hfq and RNA/Hfq/Crc assemblies (Figure 4D), the complexes were pre-formed as described above using 80 nM Hfq, 10 nM amiE\textsubscript{6ARN} RNA in the presence or absence of 480 nM Crc in a 60 \( \mu \)l reaction volume containing 12 \( \mu \)l loading dye (see above). After 2 min pre-incubation, 10 \( \mu \)l were loaded on a 4% native polyacrylamide gel and then 100 nM unlabeled amiE\textsubscript{6ARN} RNA was added. 10 \( \mu \)l samples were loaded 15, 45, and 120 sec thereafter. The gel was run continuously during the experiment.

For simultaneous detection of PrrF2 and amiE\textsubscript{6ARN} RNA (Figure 6D), PrrF2 was labeled at the 3′-end with pCp-Cy5 as mentioned above. The amiE\textsubscript{6ARN} RNA oligonucleotide labeled at the 5′-end with 6-carboxyfluorescein (6-FAM) was purchased from Sigma. Either 10 nM PrrF2-Cy5 or 100 nM FAM-amiE\textsubscript{6ARN} RNA or both were incubated in ES-buffer (see above) and 25 ng tRNA in a total volume of 10 \( \mu \)l in the absence or presence of 120 nM Hfq, or in the absence or presence of 960 nM Crc protein. The mixtures were incubated at 37°C for 15 min to allow protein-RNA complex formation. The samples were mixed immediately before loading with 2 \( \mu \)l 25% glycerol or— in case no fluorescently labeled RNA was used—with 2 \( \mu \)l loading dye (25% glycerol, 0.2 mg/l xylencyanol and bromphenol blue), and then separated on a 4% polyacrylamide gel using Tris–borate buffer.

The radioactively or fluorescently labeled bands were visualized with a phosphorImager (Molecular Dynamics).

RESULTS

Target genes of Hfq and Crc overlap

Previous studies suggested that both, Hfq and Crc, are required for tight translational repression of mRNAs, which are subjected to carbon catabolite repression (CCR) (7). Translational repression is frequently accompanied with a reduced stability and a reduced abundance of target mRNAs. Therefore, RNAseq based transcriptome analyses were performed with strains PAO1, PAO1\textsubscript{hfq—} and PAO1\textsubscript{Δcrc} with the aim to reveal overlapping mRNA targets, regulation of which is governed by both, Hfq and Crc during CCR. The strains were grown to an OD\textsubscript{600} of 1.5 in BSM complex medium. As succinate is the preferred carbon source of PAO1 it was included in the medium to establish CCR. The other C/N sources were added to induce transcription of the respective CCR-controlled genes (1,39). Except for glutamate, the uptake and/or utilization of the other compounds present in the BSM complex medium are known to be under CCR control (7,14,40–44). A \( P \)-value (adjusted for multiple testing) of 0.05 was set...
as a threshold for significance and only transcripts with a change in abundance (fold-change) of ±5 were considered in this study to select predominantly for transcripts that are stringently regulated during CCR. Applying these criteria 332 and 149 transcripts were found to be differentially abundant in PAO1/hfq- and PAO1Δcrc, respectively, when compared with PAO1. Among these were 227 and 44 non-overlapping transcripts displaying a differential abundance in either PAO1/hfq- or PAO1Δcrc when compared with PAO1 (Figure 1A; Supplementary Tables S2 and S3). In addition, four transcripts showed an opposite abundance in the PAO1/hfq- and PAO1Δcrc mutants when compared with PAO1 (Figure 1A and Supplementary Table S4). Possible reasons for the seemingly independent regulation of these transcripts by either Hfq or Crc are discussed below. In addition, 105 over-lapping transcripts were found in PAO1/hfq- and PAO1Δcrc, 55 and 46 of which were down-regulated and up-regulated, respectively (Figure 1A). The differential abundance of the majority of these transcripts was more pronounced in the absence of Hfq than in the absence of Crc (Supplementary Tables S5 and S6), which can be rationalized with our recent studies, wherein Hfq was shown to bind to target mRNAs and to obstruct ribosome loading, whereas Crc appeared to enhance the function of Hfq (7). When compared with PAO1, the most up-regulated transcripts in PAO1/hfq- and PAO1Δcrc encode proteins involved in transport and utilization of sugars and sugar alcohols (Supplementary Table S5). Given that Hfq and Crc repress CCR-controlled genes at the translational level, this can be readily explained by translational activation of the respective genes when Hfq and Crc are absent. All transcripts with decreased abundance in PAO1/hfq- and PAO1Δcrc comprise genes regulated by either of the three major quorum sensing (QS) systems LasI/R, RhII/R and Pqs or by the quorum sensing regulator QscR (Supplementary Table S6). Given that the QS systems operate in a hierarchical manner (45) it is difficult to delineate the impact of Hfq/Crc on a particular QS system. Nevertheless, the regulatory effects of Hfq/Crc on QS are most probably indirect (46,47). Taken together, when compared with the parental PAO1 strain, the respective up-regulation of CCR-controlled genes in both the PAO1/hfq- and the PAO1Δcrc strains corroborated the hypothesis that Hfq and Crc act in concert.

In vivo association of Hfq and Crc

Recent in vivo binding studies revealed that P. putida Hfq, Crc and the RNA CrcZ form a complex (11). To obtain evidence for an in vivo interaction between Hfq and Crc, an E. coli bacterial two-hybrid system (BACTH) was employed. In brief, we constructed various C- and N-terminal fusion proteins of PAO1 Hfq and Crc with the catalytic domains T18 and T25, respectively, of Bordetella pertussis adenylate cyclase. In case of an interaction between Hfq and Crc, this approach was anticipated to lead to reconstitution of functional adenylate cyclase, resulting in cAMP synthesis, which in turn is required for transcription of the lacZ gene. The combination of two variants of these fusion proteins, i.e. T25-Hfq/T18-Crc and T25-Crc/Hfq-T18, resulted in cyclase activity, indicating that Hfq and Crc interact in vivo (Figure 1B). All other combinations resulted in comparable low β-galactosidase activities as obtained for the control strain E. coli BTH101(pUT18, pKT25) (Supplementary Figure S1). Apparently, a functional interaction of the cyclase domains depended on whether the respective domains were fused to the N- or C-terminus of either Hfq or Crc. All C-terminal extensions of Crc rendered the resulting fusion proteins non-functional, indicating that C-terminal alterations in Crc might disturb the interaction with Hfq (Supplementary Figure S1). In contrast, fusions of T25 and T18 to the N- and C-terminus of Hfq, respectively, permitted an interaction with the respective N-terminal Crc fusion proteins. To ensure that endogenous E. coli Hfq does not interfere with the interaction of the PAO1 Hfq and Crc proteins, an E. coli HfqEc-T18 fusion protein was included in the assay. In contrast to T25-Crc/HfqEc-T18, co-synthesis of T25-Crc and HfqEc-T18 did not result in reconstitution of the cyclase activity, suggesting that Crc and HfqEc do not interact in this assay (Figure 1B).

To verify that Hfq and Crc interact in vivo in PAO1 a co-immunoprecipitation (co-IP) experiment using anti-Hfq antibodies was performed. Anti-Hfq antibodies were added to a PAO1 lysate and then captured with magnetic protein G-beads. The beads were eluted and the proteins were resolved on SDS-polyacrylamide gels followed by detection of Hfq and Crc by western-blotting using anti-Hfq and anti-Crc antibodies, respectively. As shown in Figure 1C, lane 1, Crc was co-immunoprecipitated together with Hfq, which was not observed with lysates of the control strains PAO1Δcrc (Figure 1C, lane 2) and PAO1Δhfq (Figure 1C, lane 3), respectively.

The association of Hfq and Crc requires RNA bound to the distal poly(A) binding side of Hfq

Crc was shown to associate with Hfq in vitro in the presence of CrcZ RNA (11). In contrast, no binding of Crc was observed in the presence of CrcZ with the distal side mutant Hfq1253 (11) that is defective in binding to CrcZ (7). To extend these studies, we performed in vitro co-IP assays with CrcZ RNA and the authentic Hfq-binding motif of the CCR-controlled amiE mRNA, which encodes aliphatic amidase (7). The amiEARN RNA (5′-AAAAAUAACAACAAGG-3′) consists of six tripartite binding motifs, which can be potentially accommodated in the six distal binding pockets of Hfq (7). In addition, poly(A)27 RNA was used, which is likewise anticipated to bind to the distal side of Hfq (48). Moreover, poly(U)14 as well as PrrF2 sRNA, both of which bind to the proximal side of Hfq (14,48) were included in the assays. PAO1 Hfq and Crc were incubated in the presence of amiEARN RNA, poly(U)14 RNA, CrcZ RNA, poly(A)27 or PrrF2 RNA (Figure 2A). Then, anti-Hfq antibodies were added to test whether Crc co-immunoprecipitates with Hfq using the magnetic bead technology. Without addition of RNA, Crc was not captured with Hfq (Figure 2A, lane 6). Crc associated with Hfq in the presence of either amiEARN RNA (Figure 2A, lane 7), CrcZ RNA (Figure 2A, lane 11) or poly(A)27 RNA (Figure 2A, lane 13) but not in the presence of poly(U)14 RNA (Figure 2A, lane 9) or PrrF2 RNA (Figure 2A, lane 15). Taken together, these studies corrob-
rate the hypothesis that Hfq and Crc form a complex in the presence of RNA bound to the distal side of Hfq.

To verify these in vitro results a HfqY25D-T18 fusion protein was included in the BACTH assay. The Y25D exchange in P. aeruginosa Hfq renders the protein variant defective in binding with its distal side to A-rich motifs (7). As shown in Figure 2B, the Y25D exchange in HfqY25D-T18 abolished the interaction with the T25-Crc protein. This experiment together with the in vitro co-IP studies supported on the one hand the idea that Hfq and Crc interact only in the presence of an RNA bound to the distal side of Hfq. Furthermore, the BACTH experiments in E. coli also suggested on the other hand that this interaction does not require specific RNA substrates provided that they bind to the distal side of Hfq.

Next, solution state nuclear magnetic resonance (NMR) spectroscopy was used to analyze the interaction between Hfq and Crc in the absence and presence of RNA. As shown in Figure 3A, upon addition of increasing amounts of Hfq-hexamer (Hfq6) only two signals showed little chemical shift perturbations on the 2D 1H-15N HSQC spectra of 15N-labelled Crc. Thus, Hfq and Crc apparently hardly asso-

Figure 1. (A) RNAseq analyses revealed an overlap between the Hfq and Crc regulon. The Venn diagram shows the number of transcripts with increased (I), decreased (D) or opposite (O) abundance in the PAO1hfq- and PAO1Δcrc mutants when compared with the PAO1 wt strain. For significance, only transcripts with a fold-change ≥ 5 and a multiple testing adjusted P-value ≤ 0.05 were considered. The corresponding transcripts with increased or decreased abundance are listed in Supplementary Table S2 (PAO1hfq- versus PAO1), Supplementary Table S3 (PAO1Δcrc versus PAO1), Supplementary Table S5 (transcripts with increased abundance affected by both, Hfq and Crc) and Supplementary Table S6 (transcripts with decreased abundance affected by both, Hfq and Crc). The transcripts showing opposite abundance (O) in the PAO1hfq- and PAO1Δcrc mutants when compared with PAO1 are listed in Supplementary Table S4. (B) In vivo association of Hfq and Crc tested with the BACTH system. N-terminal and C-terminal fusion proteins of Crc and Hfq with T18 and T25 of adenylate cyclase were constructed as described in Supplementary Text S1. The E. coli strain BTH101 was co-transformed with plasmids encoding the respective fusion proteins as indicated below the blue bars. Functional adenylate cyclase is only reconstituted when Crc and Hfq interact with each other, which is reflected by β-galactosidase production. White bar, background production of β-galactosidase in E. coli BTH101(pUT18, pKT25) harboring the parental plasmids. Black bar, co-synthesis of T25-Crc and HfqEc-T18 did not result in reconstitution of the cyclase activity. The results of three independent experiments were averaged and are shown as mean ± standard deviation. (C) In vivo co-IP of Hfq and Crc. The experiment was performed with lysates of strains PAO1 (wt) (lane 1), PAO1Δcrc (lane 2), and PAO1Δhfq (lane 3) and anti-Hfq specific antibodies and magnetic protein G beads. The in vivo association of Hfq with Crc was visualized by western-blot analysis using either anti-Crc or anti-Hfq specific antibodies. The upper nitrocellulose strip was overexposed to visualize Crc. Lane 4, 5 pmol of either purified Crc or Hfq protein were loaded.
Figure 2. The association between Hfq and Crc requires RNA binding to the distal side of Hfq. (A) In vitro co-IP experiments were performed with purified components as indicated on top of the Figures, anti-Hfq specific antibodies and magnetic protein G beads. The in vitro association of Hfq with Crc was visualized by western-blot analysis using anti-Crc or anti-Hfq specific antibodies. Lanes 1 and 2, 4 pmol Hfq and 12 pmol Crc were loaded, respectively. Lanes 3–5, control experiments in the absence of both proteins (lane 3) or presence of either only Hfq (lane 4) or Crc (lane 5). Lanes 6–16, co-IP with anti-Hfq antibodies in the presence of Hfq and Crc (lanes 7, 9, 11, 13, and 15) and in the absence of Crc lanes 8, 10, 12, 14, and 16), respectively, with no RNA added (lane 6) and in the presence of amiE6ARN (lane 7), poly(U)14 (lane 9), CrcZ (lane 11), poly(A)27 (lane 13) and PrrF2 sRNA (lane 15), respectively. (B) BACTH analysis of the Crc-Hfq Y25D interaction in E. coli strains BTH101(pUT18,pKT25) (white bar), BTH101(pHfq-T18,pKT25-Crc) (black bar) and BTH101(pHfqY25D-T18,pKT25-Crc) (yellow bar), respectively. The results of three independent experiments were averaged and are shown as mean ± standard deviation.

Crc enhances the lifetime of Hfq/RNA complexes

We next studied whether the presence of Crc increases the affinity of Hfq for amiE6ARN RNA by employing EMSA assays. The amiE6ARN RNA was labelled at the 5'-end with \( \gamma^{32P} \)-ATP and 10 nM were incubated with increasing amounts of Hfq in the presence or absence of Crc-protein. As shown in Figure 4A, in the absence of Crc (lanes 1–5) an observable band shift was obtained when Hfq was added in 8-fold molar excess over \( ^{32P}-amiE6ARN \) RNA. In contrast, in the presence of Crc (Figure 4A, lanes 6–10) a shift of the \( ^{32P}-amiE6ARN \) RNA to the protein bound state (A*HC) occurred with the results shown in Figures 2A and 3A. As observed previously (24), Crc alone did not bind to amiE6ARN RNA (Supplementary Figure S2), which again showed that the RNA binding protein in the Hfq/Crc/RNA complex is Hfq.
Kd = 134.6 ± 10.2 nM

Figure 3. In vitro association between Hfq and Crc in the presence of RNA. (A) Overlay of the 2D 15N–1H BEST-TROSY HSQC recorded before and after addition of unlabelled Hfq6. The resulting spectra are colored according to the molar ratio of Crc: Hfq6 (black 1:0; red 1:1; blue 1:2; magenta 1:3). NMR signals that experienced chemical shift changes are boxed. (B) Overlay of the 2D 13C–1H HMQC spectra of 13C-methyl-labelled Crc recorded before (black spectra) and after addition of equimolar amounts of the unlabelled Hfq6/amiE6ARN complex (red spectra). (C) MST analysis with 30 nM labelled Hfq6, 30 nM amiE6ARN and increasing amounts of Crc. (D) MST analysis with 30 nM labelled Hfq6 and increasing amounts of Crc. Data from two independent experiments are shown as mean ± standard deviation. Thermophoresis/T-jump analysis is shown. LED power of 90% and MST power of 60% were used.

Figure 4. (A) Overlay of the 2D 15N–1H BEST-TROSY HSQC recorded before and after addition of unlabelled Hfq6. The resulting spectra are colored according to the molar ratio of Crc: Hfq6 (black 1:0; red 1:1; blue 1:2; magenta 1:3). NMR signals that experienced chemical shift changes are boxed. (B) Overlay of the 2D 13C–1H HMQC spectra of 13C-methyl-labelled Crc recorded before (black spectra) and after addition of equimolar amounts of the unlabelled Hfq6/amiE6ARN complex (red spectra). (C) MST analysis with 30 nM labelled Hfq6, 30 nM amiE6ARN and increasing amounts of Crc. (D) MST analysis with 30 nM labelled Hfq6 and increasing amounts of Crc. Data from two independent experiments are shown as mean ± standard deviation. Thermophoresis/T-jump analysis is shown. LED power of 90% and MST power of 60% were used.

curred already when Hfq was added in 2-fold molar excess over the RNA (Figure 4A, lane 7). In addition, MST assays were performed with 30 nM labelled Hfq6 and increasing amounts of amiE6ARN RNA in the absence or presence of Crc protein. The Kd-value increased from 43.2 ± 2.3 nM in the absence of Crc (Figure 4B) to 33.9 ± 1.7 nM in the presence of Crc (Figure 4C). As the increase in the affinity was rather moderate this result rather argued against the idea that the presence of Crc results in a significant affinity enhancement of Hfq for the substrate.

We therefore asked whether the presence of Crc increases the stability of the Hfq/Crc/RNA complex. Pre-formed 32P-amiE6ARN/Hfq or 32P-amiE6ARN/Hfq/Crc complexes were incubated for different times with 100 nM unlabelled competitor amiE6ARN RNA (10-fold molar excess over the labelled RNA), and then loaded after 15, 45 and 120 s on a continuously running native polyacrylamide gel. The apparent release of Hfq from the binary complex (A*H) was already observed after 15 s (Figure 4D, lane 3). In contrast, the Hfq/Crc/amiE6ARN RNA complex was significantly more stable. Even after 120 s the majority of the labelled RNA was still present in the A*HC complex (Figure 4D, lane 10). Taken together these experiment indicated that
Figure 4. The presence of Crc stabilizes the Hfq/RNA complex. (A) Electrophoretic mobility shift assays (EMSA) with $^{32}$P-amiE$_{6ARN}$ RNA and increasing amounts of Hfq in the absence and presence of Crc. Lane 1, electrophoretic mobility of $^{32}$P-amiE$_{6ARN}$ RNA (A*) in the absence of proteins. Lanes 2–5, $^{32}$P-amiE$_{6ARN}$ RNA/Hfq complex formation (A*H) with increasing concentrations of Hfq. Lanes 6–10, $^{32}$P-amiE$_{6ARN}$ RNA/Crc/Hfq complex formation (A*HC) with increasing concentrations of Hfq. (B and C) MST analysis with 30 nM labelled Hfq and increasing concentrations of amiE$_{6ARN}$ RNA in the absence (B) and presence of 360 nM Crc (C). The results represent data from two independent experiments and are shown as mean ± standard deviation. Thermophoresis/T-jump analysis is shown. LED power of 90% and MST power of 60% were used. (D) Hfq-RNA dissociation in the absence and presence of Crc. $^{32}$P-amiE$_{6ARN}$ RNA was pre-incubated with Hfq (A*H complex; lanes 2–5) or with Hfq and Crc (A*HC complex; lanes 7–10). Then, unlabelled amiE$_{6ARN}$ competitor RNA was added for the times given in seconds followed by electrophoresis on a native polyacrylamide gel. Lane 1, electrophoretic mobility of $^{32}$P-amiE$_{6ARN}$ RNA (A*) in the presence of unlabelled $^{32}$P-amiE$_{6ARN}$ RNA. Lane 2, electrophoretic mobility of $^{32}$P-amiE$_{6ARN}$ RNA (A*) in the presence of unlabelled $^{32}$P-amiE$_{6ARN}$ RNA and Crc. The concentrations of the ligands are given at the right.

The presence of Crc enhances the lifetime of the Hfq/RNA interaction.

The interaction of Crc with RNA and Hfq revealed by protein-RNA and protein-protein cross-linking

A possible explanation for the RNA-induced formation of the Hfq/Crc/RNA complex was that Crc exploits multivalency as a means to stabilize Hfq/Crc/RNA complexes. In other words, even though interactions between Crc and RNA (24) and Crc and Hfq were not observed in the absence of RNA (Figures 2A and 3D), simultaneous interactions of Crc with both Hfq and RNA might considerably increase the avidity of the complex for RNA. We therefore used UV-induced cross-linking in combination with mass spectrometry to detect such interactions in the UV-cross-linked Hfq/Crc/amiE$_{6ARN}$ complex. These analyses revealed that amino-acid residues Y$_{94}$ and K$_{236}$ in Crc interact with U/C and U bases, respectively, of the amiE$_{6ARN}$ 18-mer RNA (Supplementary Figure S3A and B). Y$_{94}$ and K$_{236}$ are situated diametrically opposed on the surface of Crc (Figure 5A and B).

The protein-protein cross-linking experiments with bis(sulfosuccinimidyl)suberate (BS3) revealed four interactions between Crc and Hfq: CrcK$_{77}$-HfqK$_{17}$, CrcK$_{122}$-HfqM$_{1}$, CrcK$_{124}$-HfqK$_{3}$ and CrcK$_{236}$-HfqK$_{3}$, respectively (Supplementary Table S7; Figure 5A and B; Supplementary Figure S4). Hfq M$_{1}$, K$_{3}$ and K$_{17}$ are situated on the proximal surface of Hfq (Supplementary Figure S4; (49)). To further validate that the Crc-Hfq interactions are only occurring in the presence of RNA, the same experiment was performed in the absence of RNA. As shown in Supplementary Table S8, no interactions between Crc and Hfq were identified under these conditions.

It was rather unexpected to detect only a few cross-links between Hfq and Crc. A most likely explanation is intrinsic
Figure 5. Amino acid residues in Crc implicated in Hfq and RNA interactions. The C-side and N-side of Crc are termed according to the localization of the N- and C-terminus, respectively. (A and B) Ribbon diagram (top), surface representation (middle) and electrostatic surface potential (bottom) of the Crc C-side (24) containing the basic patch (A) and the N-side opposed to it (B). The positions of the N- and C-termini are depicted in the ribbon diagrams (top) and are colored in dark blue (N-terminus) and green (C-terminus) in the surface structure (middle), respectively. K residues that were found to be cross-linked in the Hfq/Crc amiE_6ARN complex are depicted in light blue. Residues in Crc that were found to interact with amiE_6ARN RNA are highlighted in yellow. Amino acid residues that were found to be altered in the PAO1 ΔcrcZ sup mutants are highlighted in red. α-helices are colored in black, β-strands in light grey, and coils in dark grey, respectively. Image visualization was performed with Chimera (38). The electrostatic surface potential was calculated by Coulomb’s law and visualized by Chimera (38). The electrostatic potential ranges from -10 (red) to +10 (blue) kcal/(mol*e) at 298K.

to the method used. It turned out that Hfq was not well digested by trypsin and Lys-C, which probably resulted in a very limited number of Hfq-specific peptides for mass spectrometric analyses. In any case, these cross-linking experiments showed that Crc binds to RNA as well as to Hfq, indicating that the protein exploits several interactions.

In addition to the above mentioned interaction, several other BS3 induced cross-links were found originating from Crc derived peptides. The cross-links between Crc residues K_{77}, K_{101}, K_{124} and K_{135} (Supplementary Table S7), which are located on the same side as the RNA binding residue Y_{94} (Figure 5A), were predominantly observed. In addition, interactions between several other Crc specific residues occurred. To distinguish between possible intra- and intermolecular interactions, the intramolecular distances between the crosslinked residues of Crc (PDP: 4JG3) were calculated with the distance calculation tool of the USCF Chimera package (Supplementary Table S7; (38)). The spacer arm length of BS3 is 11.4 Å. With the exception of the Crc_{K77-Crc{K101}} crosslink, which most likely results from the spatial proximity of both residues in Crc (24) all other cross-linked residues are further apart (Supplementary Table S7), which favors the idea that the observed Crc-Crc interactions are intermolecular. In addition, a number of apparent intermolecular Crc-Crc cross-links were also noticeable when BS3 cross-linking was performed with Hfq and Crc in the absence of RNA (Supplementary Table S8). However, they occurred to a lower extent when compared with those observed in the Hfq/Crc/amiE_{6ARN} complex. There is so far no evidence for oligomerization of Crc monomers from structural (24) and biochemical studies (Supplementary Figure S5C). Thus, we can only speculate that weak interactions occurred under these experimental conditions, which are not revealed by other means.

These studies raised the question whether the size of the Hfq/Crc/RNA complex is larger than expected form a 1:1:1 stoichiometry. The size of the complex was assessed by SEC-MALS (size exclusion chromatography com-
bined with multi-angle laser light scattering; Supplementary Text S1). In solution, the measured molecular mass of the Hfq/Crc/amiEΔARN complex was determined with 219 800 g mol⁻¹ (Supplementary Figure S5A). Given that the Hfq hexamer and the Crc monomer are 53.7 kDa (Supplementary Figure S5B) and 29.6 kDa (Supplementary Figure S5C) proteins in solution, respectively, and that the amiEΔARN RNA accounts for ~ 6100 g mol⁻¹, the size of the complex indicates that several Hfq and/or Crc molecules are present. The presence of at least two Crc monomers in the Hfq/Crc/amiEΔARN complex is indicative from some cross-links obtained with BS3 as the Crc aa residues K₁₁₅, K₁₃₅, and K₁₅₆ were found to be auto-linked (Supplementary Table S7).

Genetic dissection of the Hfq–Crc interaction

To gain further insights into the Hfq–Crc interactions, we made use of the observation that a PAO1ΔcrcZ strain is defective in utilization of a number of carbon sources (Supplementary Table S9). This can be explained by the absence of the Crc complex preventing relief of Hfq/Crc-mediated repression of gene functions required for metabolism of these carbon sources (7). PAO1ΔcrcZ revertants that regained the ability to grow on either histidine, alanine, acetamide or mannitol were screened for mutations in the hfq and crc genes with the rationale to isolate variants that lost the ability to interact with either Hfq or Crc. The PAO1ΔcrcZ revertants were analyzed by means of colony PCR followed by DNA sequencing of the hfq and crc genes (Supplementary Text S1). This analysis revealed 25 intragenic mutations in the crc gene, one in the hfq gene and two extragenic suppressor mutations, the latter of which were not further characterized (Supplementary Table S10).

The mutation in the hfq gene resulted in an exchange of P₆₄ to S (Supplementary Table S10). As shown in Supplementary Figure S4A and B, P₆₄ is located proximal to the flexible C-terminus and could affect its lateral orientation (49–51).

Among the twenty-five mutations detected in the crc coding region were such that led to the generation of premature stop codons, deletions, frame-shifts or to an extension of the reading frame (Supplementary Table S10). These were not further studied. Six missense mutations resulting from single nucleotide changes in crc were identified, which resulted in amino acid substitutions at five different positions in Crc (Supplementary Table S10). Three (P₇₆, R₁₄₁, T₂₂₅) of the five altered amino acid residues are located on the C-side of Crc (Figure 5A), and are fully conserved in the Crc proteins of the sequenced Pseudomonadaceae (not shown). V₁₀₂ is close to the surface and in the same region as the other surface exposed residues, whereas L₁₄₀ is buried inside the globular structure of Crc (Figure 5A and B). V₁₀₂ and L₁₄₀ are not fully conserved in the Crc proteins of the Pseudomonadaceae but the variations comprise amino-acid residues with strongly similar properties (not shown). It is also worth noting that K₁₀₉ and K₇₇, which were frequently found to cross-link with other Crc residues (Supplementary Table S7), as well as Y₉₄, which was cross-linked to RNA, are as well located in the same region on the Crc surface (Figure 5A and B).

For further analysis we focused on the following revertants: PAO1ΔcrcZsup₃₄ (HfqP₆₄S), PAO1ΔcrcZsup₂₇ (CrcV₁₀₂E), PAO1ΔcrcZsup₂₉ (CrcT₁₄₉R), PAO1ΔcrcZsup₆ (CrcP₁₇₆), PAO1ΔcrcZsup₅ (CrcT₁₂₅S) and PAO1ΔcrcZsup₉ (CrcR₁₄₁L). Since the PAO1ΔcrcZ revertants were isolated after growth on different carbon-sources, we first tested whether their phenotype was independent on the respective carbon source used for their selection. All mutants were able to grow on histidine, alanine, acetamide and mannitol (Supplementary Figure S6). We therefore hypothesized that the respective Hfq and Crc variants lead to a general alleviation of CCR.

To corroborate this, we tested the proficiency of the Hfq and the Crc mutant proteins to repress translation of an amiE::lacZ reporter gene during CCR (7). The strains PAO1, PAO1ΔcrcZ, PAO1Δhfg, PAO1Δcrc and the six PAO1ΔcrcZ mutant strains were transformed with plasmid pME9655, encoding a translational amiE::lacZ fusion gene, and with the empty vector pME4510 (Supplementary Table S11). The strains were subjected to CCR by cultivation in BSM medium supplemented with 40 mM succinate and 40 mM acetamide, the latter of which was added to induce transcription of the amiE::lacZ gene. When compared with the PAO1ΔcrcZ (pME9655, pME4510) strain, the translation of the amiE::lacZ reporter gene was increased in all PAO1ΔcrcZ revertants, albeit to a lower level when compared with the PAO1Δcrc (pME9655, pME4510) and PAO1Δhfg (pME9655, pME4510) strains (Supplementary Table S11). This indicated a partial loss of function of the Crc variants and of the HfqP₆₄S mutant protein with regard to Hfq/Crc/RNA complex formation. The complementation of the crc and hfg alleles of the PAO1ΔcrcZ revertants with plasmid encoded wild-type copies of crc (pME4510crcFLAG) and hfg (pME4510hfgFLAG) (Supplementary Table S11), respectively, resulted in full repression of amiE::lacZ translation, which clearly attributed the PAO1ΔcrcZ suppressor phenotype to the crc and hfg missense alleles. On the other hand, it suggested that the relief of Hfq/Crc-mediated repression observed with the revertants (Supplementary Table S11) is attributable to an impairment in the Hfq/Crc/RNA interaction.

Next, the BACTH assay was employed to further test whether the mutant proteins encoded by the different crc and hfg missense alleles are impaired in Hfq/Crc interactions. As shown in Supplementary Figure S7, none of the T25-Crc variant proteins interacted with Hfq-T18. In addition, the interaction of HfqP₆₄S-T18 with T25-Crc was apparently impaired. Taken together, these experiments indicated that the respective Hfq and Crc variants are defective in Hfq/Crc complex formation, which can readily explain the observed alleviation of CCR in the PAO1ΔcrcZ revertants.

As discussed below, although we cannot distinguish whether these single amino acid changes affect binding of Crc to Hfq or RNA, it seems worth noting that they are located in close proximity to Y₉₄, which cross-linked with RNA (Figure 5A). The HfqP₆₄S mutant protein was apparently impaired in Hfq/Crc/RNA complex formation (Supplementary Table S11) and Crc cross-linked to Hfq K₁₇ and K₁₇. The latter residues are part of the N-terminal α-helix situated on the proximal side of Hfq (Supplementary Figure
SSA), and, as mentioned above, HfqP64S is located proximal to the flexible C-terminus and most likely affects its lateral orientation on the proximal side. Therefore, we next asked whether Crc might interfere with binding of a sRNA to the proximal side of Hfq.

**Crc interferes with binding of a sRNA to the proximal side of Hfq**

To address this, we made use of our recent observation that the PAO1 sRNA PrrF1–2 binds to the proximal side of Hfq (14). First, MST was used to test whether the presence of Crc interferes with PrrF2 binding. As shown in Figure 6A, the $K_d$ of Hfq for Cy5 labelled PrrF2 was determined with $\sim 6.7 \pm 0.3$ nM. The $K_d$ of Hfq for PrrF2-Cy5 did not significantly change in the presence of a 12-fold molar excess of amiE$_{6ARN}$ RNA (Figure 6B), the latter of which was shown to bind to the distal side of Hfq (7). However, when Crc was additionally included in the assay, the $K_d$ of Hfq for PrrF2-Cy5 declined to 35.0 $\pm$ 3.0 nM (Figure 6C), indicating that the formation of the Hfq/Crc/amiE$_{6ARN}$ complex interferes with binding of the sRNA to Hfq. Next, EMSA assays were performed with PrrF2-Cy5 and amiE$_{6ARN}$FAM in the presence of Hfq as well as in the presence of both, Hfq and Crc. As shown in Figure 6D, lane 5, in the presence of Hfq alone, both RNAs were bound to Hfq. When Crc was additionally included in the assay, a supershifted species of Hfq/Crc/6-FAM-amiE$_{6ARN}$ was observed, but a quaternary complex composed of Hfq/Crc/6-FAM-amiE$_{6ARN}$/PrrF2-Cy5 was not observed (Figure 6D, lane 7). Although the latter experiment might be interpreted as showing that Crc interferes with PrrF2 binding to the Hfq/Crc/6-FAM-amiE$_{6ARN}$ complex, the experimental set up does not exclude the possibility that Hfq binds to either amiE$_{6ARN}$ or PrrF2.

Therefore, we sought to verify this observation in vivo by asking whether Crc can interfere with Hfq-mediated and PrrF1–2 dependent translational repression of antR mRNA (14). The $\beta$-galactosidase activity conferred by a translational antR::lacZ reporter gene expressed from plasmid pTLantR was monitored in strain PAO1 ΔcrcΔcrcZ. As both Hfq and Crc were shown to impact on the level of CrcZ RNA (52), the double mutant was chosen to exclude the possibility that CrcZ interferes with Hfq-mediated and PrrF1–2 dependent riboregulation of antR mRNA (14). In addition to plasmid pTLantR, the strain harbored either the parental vector pME4510 or plasmid pME4510 crcFlag, which permitted over-production of a CrcFlag variant (Figure 6E top panel, lane 2). When compared with strain PAO1 ΔcrcΔcrcZ (pTLantR, pME4510), overexpression of crcFlag in strain PAO1 ΔcrcΔcrcZ (pTLantR, pME4510 crcFlag) resulted in de-repression of antR::lacZ translation (Figure 6E). As the antR promoter activity was unaffected by Crc under these conditions (Supplementary Figure S8), this observation agreed with the notion that Crc can interfere with binding of PrrF2 to the proximal side of Hfq. The RNAseq analyses lend support to these observations. They revealed four transcripts that showed an opposite abundance in PAO1hfq– and PAO1Δcrc when compared with PAO1. Among them are the antABC transcripts, (Supplementary Table S4), which are positively regulated by the transcription factor AntR. These genes were up-regulated in PAO1hfq–, consistent with the finding that negative translational regulation of the antR mRNA by the sRNA PrrF1–2 is abrogated in the absence of Hfq (14). In opposite, the antABC transcripts showed a decreased abundance in PAO1Δcrc. This in turn can be reconciled with the experiment shown in Figure 6E, sindicating that translational repression of antR by Hfq and PrrF1–2 is more efficient in the absence of Crc.

**DISCUSSION**

The Hfq/Crc/sRNA complex, a multipart ensemble

Genetic, biochemical and biophysical studies showed that efficient Hfq/Crc/sRNA complex formation requires binding of an RNA molecule to the distal poly(A) binding side of Hfq (Figures 2 and 3). We infer from the BACTH assays carried out in E. coli (Figure 1B), that the nature of the distal bound RNA does not matter provided that it contains an A-rich recognition motif for Hfq. The bound RNA could bring about the Hfq/Crc interaction by inducing conformational changes that permit the interaction with Crc. Although there is some evidence from NMR studies for a cross-talk between the distal and proximal sides of Hfq upon poly(A) binding (53), other biophysical studies suggested that the core region of E. coli Hfq is rather rigid (48,51). Given that Crc was shown to cross-link with both, Hfq and RNA, we rather favor the idea that Crc exploits several interactions as a means to assemble into Hfq/Crc/sRNA complexes. As Crc does not detectably interact with Hfq alone (Figure 3D), and the affinity of Hfq is comparable to that of the Crc/Hfq/sRNA complex for the distal bound RNA (Figure 4B and C), we hypothesize that the RNA in the Hfq-Crc/sRNA complex serves as a toehold for Crc assembly. Although the presence of Crc did not significantly enhance the affinity of Hfq for the RNA (Figure 4C), the simultaneous interactions of Crc with both binding partners result in an Hfq/Crc/sRNA assembly with increased lifetime when compared with the Hfq/sRNA complex alone (Figure 4D). This in turn can explain the function of Crc in Hfq-mediated translational repression of target mRNAs during CCR (7,11). Hence, Crc is the first proteinaceous factor shown to modulate Hfq-mediated RNA binding.

The intermolecular distances between the cross-linked and surface exposed K residues in Crc as well as between the auto-linked K residues 77, 135, 155 and 236 (Supplementary Table S7) are indicative for intermolecular interactions between Crc entities, which is in agreement with the observation that Hfq, Crc and RNA do apparently not assemble with a 1:1:1 stoichiometry (Supplementary Figure S5). It is also worth noting that the crc suppressor mutations affecting amino acid residues 76 (P76L) and 102 (V102E) are in juxtaposition to K77 and K101 that were found to cross-link frequently with Crc residues 135/155 and 135, respectively (Supplementary Table S7). In addition, CrcK77 cross-linked with HfqK17. Thus, the loss of function of these mutant proteins with regard to the Hfq/Crc mediated repression of amiE::lacZ translation (Supplementary Table S11) and Hfq/Crc interaction (Supplementary Figure S7) could result from their negative effect on both Hfq/Crc and Crc/Crc interactions.
Figure 6. Crc affects binding of the sRNA PrrF2 to Hfq. MST analyses with 40 nM PrrF2-Cy5 RNA and (A) increasing concentrations of Hfq, (B) increasing concentrations of Hfq in the presence of 500 nM amiE6ARN, and (C) increasing concentrations of Hfq in the presence of 500 nM amiE6ARN and 1 μM Crc. The results represent data from two independent experiments and are shown as mean ± standard deviation. Thermophoresis/T-jump analysis is shown. LED power of 90% and MST power of 60% were used. (D) PrrF2 sRNA does not bind to the Hfq/Crc/amiE6ARN RNA complex. EMSA with 10 nM Cy5-labelled PrrF2 RNA (red bands) and 100 nM 6-FAM-labelled amiE6ARN RNA (green bands) in the absence of presence of Hfq and Crc. Lane 1, electrophoretic mobility of 6-FAM-amiE6ARN RNA (A*). Lane 2, electrophoretic mobility of PrrF2-Cy5 RNA (P*). Lanes 3 and 4, electrophoretic mobility of 6-FAM-amiE6ARN RNA (A*H; middle panel) and PrrF2-Cy5 RNA (P*H; upper panel), respectively, in the presence of 120 nM Hfq. Lane 5, electrophoretic mobility of PrrF2-Cy5 RNA and 6-FAM-amiE6ARN RNA in the presence of 120 nM Hfq. As shown in the superimposition (bottom panel) both RNAs are in complex with Hfq (P*HA*). Lane 6, electrophoretic mobility of 6-FAM-amiE6ARN RNA in the presence of 120 nM Hfq and 960 nM of Crc (A*HC). Lane 7, electrophoretic mobility of PrrF2-Cy5 RNA and 6-FAM-amiE6ARN RNA in the presence of both, 120 nM Hfq and 960 nM Crc. As shown in the superimposition PrrF2-Cy5 RNA is not part of the Hfq/Crc/6-FAM-amiE6ARN (A*HC) RNA complex. Only the Hfq bound state (P*H) is observed. (E) The strains PAO1ΔcrcΔacrZ(pTLantR, pME4510) (orange bar) and PAO1ΔcrcΔacrZ(pTLantR, pME4510creFlag) (blue bar) were grown in BSM-succinate medium. Samples were withdrawn at an OD600 of 2.0. The bars represent the β-galactosidase values conferred by the plasmid pTLantR encoded translational antR::lacZ fusion in the presence or absence of ectopic crcFlag expression, respectively. The error bars represent standard deviations from three independent experiments. Top panel, CreFlag, S1, PrrF2 and 5S rRNA levels in strains PAO1ΔcrcΔacrZ(pTLantR, pME4510) (lane 1) and PAO1ΔcrcΔacrZ(pTLantR, pME4510creFlag) (lane 2). The CreFlag levels were determined by western-blot analysis using anti-Crc antibodies. Immunodetection of ribosomal protein S1 served as a loading control. The PrrF2 and 5S rRNA (control) levels were determined by Northern blotting.
As we have identified only two cross-linked amino-acids on the opposite sides of Crc that interacted with RNA (Figure 5A and B), it is not possible to delineate a RNA binding surface. In addition, as amiE_{6ARN} RNA contains only one U-nucleotide, we cannot distinguish whether two RNA molecules are bound on either side of Crc or whether they are bound to two Crc proteins. The electrostatic surface potential of Crc revealed a basic patch on the C-side of the protein (Figure 5A, bottom). Crc Y_{94} that cross-linked with amiE_{6ARN} RNA as well as the amino acid exchanges T_{225}/I_{R_{141}} C/S identified in the genetic screen (Supplementary Table S10) are in close proximity or within this basic patch. The C-terminus of Crc is situated on top of the basic patch. The crc sup2a4 mutation altered the C-terminus in that it resulted in a 21 amino acid extension (Supplementary Table S10). Similarly, C-terminal extensions with either the T18 or the T25 domain of the adenylate cyclase rendered Crc inactive in interacting with Hfq in the BACTH system (Supplementary Figure S1). On the other hand, one of the most prominent Crc–Crc intermolecular cross-links (K_{101}/K_{155}) is found in this region. It seems worthwhile to further address the question whether the C-side is involved in RNA binding as well as in Crc–Crc interactions.

Only four intermolecular cross-links, Hfq_{M1}/Crc_{K122}, Hfq_{K3}/Crc_{K124}, Hfq_{K3}/Crc_{K236}, and Hfq_{K17}/Crc_{K77} were obtained between Hfq and Crc. (Figure 5; Supplementary Figure S4; Supplementary Table S7). As mentioned above, this can most likely be attributed to the stability of Hfq in the presence of the proteases trypsin/Lys-C. Hfq also displayed complete resistance to the proteases pepsin and chymotrypsin. Thus, there may be more interactions between both proteins that escaped our analyses. M_{1}, K_{3} and K_{17} are located on the proximal site of Hfq (Supplementary Figure S4). The hfq sup34 mutation resulted in an exchange of P_{64} to S. Hfq_{P64} represents the last amino acid of the conserved core of Hfq and precedes the C-terminus, which is most likely intrinsically unstructured (51). The C-terminus of Hfq seems to extend laterally away from the proximal side of Hfq (51, 54, 55). Thus, the Hfq_{P64} exchange might impact on the spatial orientation of the C-terminus and thus affect Crc binding. In any case, (i) RNAs binding to the proximal side did not result in Hfq/Crc/RNA complex formation (Figure 2A), (ii) a quaternary Hfq/Crc/6-FAM-amE_{6ARN}/PrrF2-Cy5 complex was not observed (Figure 6D), and (iii) Crc apparently interfered with Hfq-mediated and PrrF1–2-dependent riboregulation of antR mRNA. We interpret these results as showing that PrrF1–2 and Crc binding to Hfq is exclusive. Whether this also holds for other sRNAs remains to be studied. Currently efforts are underway to elucidate the composition and architecture of the Hfq/Crc/RNA ensemble by means of structural biology.

Physiological implications for modulation of Hfq function by Crc

The observed differences in the transcriptomes between wild-type strains and the isogenic hfq deletion mutants grown in different media ((46, 57); PRJEB22802) established Hfq as a pleiotropic regulator in Pseudomonas impacting on metabolism (7), establishment of virulence traits (10, 56, 57) including quorum sensing (46, 47) as well as on certain stress responses (58). As anticipated from a concerted action of Hfq and Crc, the transcriptome analyses revealed an overlap between the Hfq and the Crc regulon (Supplementary Tables S5 and S6). However, given the criteria used for the RNAseq analysis a number of non-overlapping transcripts displayed a differential abundance in either PAO1/hfq- or PAO1Δcrc when compared with PAO1 (Figure 1A; Supplementary Tables S2 and S3). Here, the Hfq regulon is larger than the Crc regulon (Figure 1A). This might be explained in light of the multiple tasks of Hfq, which also involves canonical riboregulation with sRNAs (14, 15). Therefore, it may not be surprising that the genetic screen did not reveal several hfq suppressor mutants. On the hand, the opposite abundance of transcripts in PAO1/hfq- and PAO1Δcrc, as exemplified by the antABC transcripts (Supplementary Table S4), most likely results from the interference of Crc with Hfq and sRNA-mediated riboregulation. Moreover, Crc is apparently involved in regulating a sub-set of Hfq regulated genes (Figure 1A), which might explain the higher number of suppressor mutations found in the cerc gene. However, it should also be noted that for significance only transcripts with a fold-change of ± 5 were considered. By lowering this threshold the number of overlapping genes was obviously increasing (not shown). Nevertheless, further efforts are necessary to understand how Crc impacts on transcripts that are not concurrently subject to regulation by Hfq.

A puzzling aspect of the study is that the affinity of Hfq for the sRNA PrrF2 is higher (Figure 6A) than the affinity of Crc for the Hfq/amE_{6ARN} complex. (Figure 3C). We hypothesize that this ensures that sRNA-mediated regulation of stress responses is put into effect when required, while the increased stability of the Hfq/Crc/RNA complexes (Figure 4D) permits maintenance of CCR, i.e. it would safeguard that Crc prioritizes the function of Hfq toward optimal carbon utilization. This working model would require more free Hfq than Crc in the cell. We have argued that in the presence of a preferred carbon source only a few other catabolites may induce concomitant transcription of the corresponding catabolic genes (7). In addition, translational repression during CCR of catabolic genes other than those required for the breakdown of the preferred carbon source appears to lead to degradation of the corresponding mRNAs (7), and thus most likely to recycling of Hfq. Therefore, CCR control may not require vast amounts of Hfq. The intracellular concentration of Hfq has been calculated with ~2160 ± 56 Hfq6 during growth in BSM-succinate medium at an OD_{600} of 2.0 (7). This is comparable with ~2350 ± 481 Crc monomers per cell determined under the same conditions (Supplementary Figure S9). The Crc levels appear not to vary with growth phase or with the carbon source (8), which indicates that Hfq6 and Crc-monomers are present in ~ equimolar quantities in the cell. However, given the size of the Hfq/Crc/RNA complex (Supplementary Figure S3A) and the number of intermolecular cross-links observed for Crc (Supplementary Table S7) one might speculate that the Hfq/Crc/RNA complex involves more Crc-monomers than Hfq6. In such a scenario enough Hfq6 might be free for stress-induced riboregulation with sRNAs during CCR.
SUPPLEMENTARY DATA
Supplementary Data are available at NAR online.

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