Sequestration of Hfq by the non-coding RNA CrcZ affects biofilm formation and the susceptibility to antibiotics in *Pseudomonas aeruginosa*

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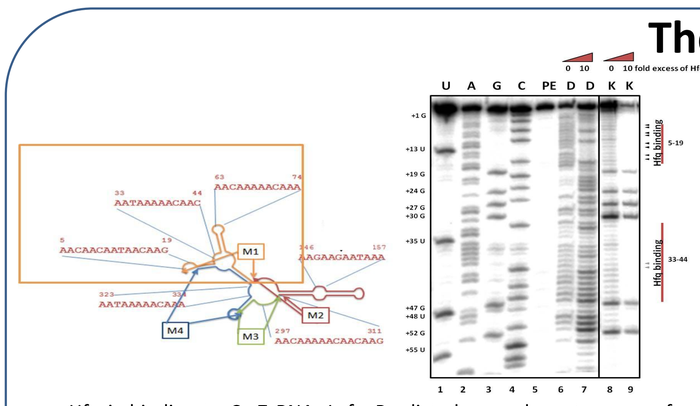
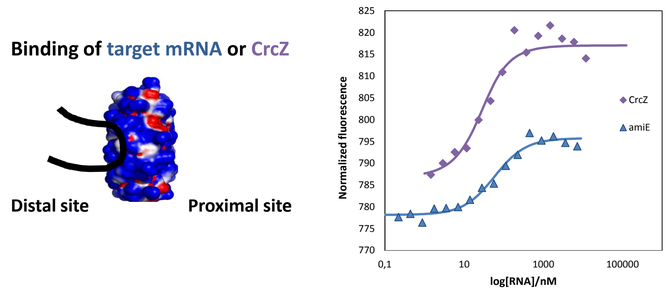
Pseudomonas aeruginosa is a major cause of nosocomial infections and a persistent pathogen in the lungs of individuals afflicted with cystic fibrosis, where it can form anaerobic biofilms that show increased tolerance towards many antibiotics. Transcriptome studies revealed that the level of the ncRNA CrcZ was highly increased in anoxic biofilms grown in synthetic cystic fibrosis sputum medium. We show that Hfq and CrcZ besides regulating carbon catabolite repression, impact on biofilm formation and the susceptibility of *P. aeruginosa* to different antibiotics.

The ncRNA CrcZ acts as a decoy for Hfq in *Pseudomonas aeruginosa*

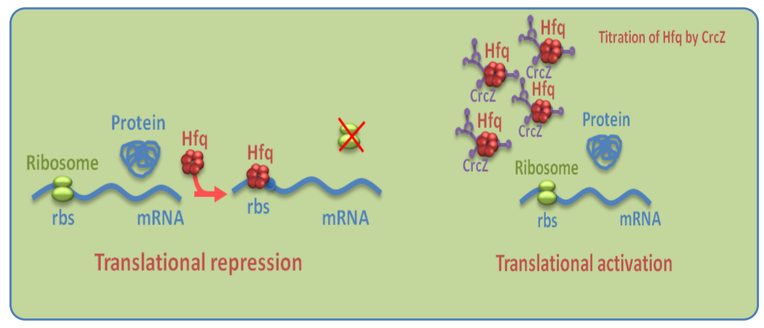


Hfq is binding to CrcZ RNA. Left: Predicted secondary structure of CrcZ RNA with 6 potential A-rich Hfq binding sites. Right: Hfq binding to CrcZ revealed by chemical probing.



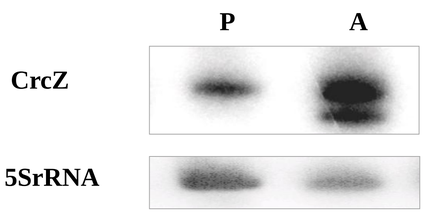
Kd (CrcZ)= 23.7 0,139 nM
Kd (*amiE*) = 65.1 0.03 nM

The CrcZ RNA binds to Hfq with high affinity. Left: *P. aeruginosa* Hfq protein interacting with mRNA or CrcZ (black). Right: K_d of Hfq for CrcZ RNA and for *amiE* mRNA revealed by microscale thermophoresis.



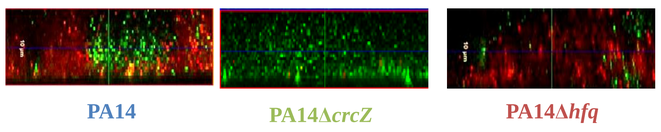
Simplified model for "regulation" of Hfq by the regulatory RNA CrcZ in *Pseudomonas aeruginosa*. Hfq (red hexamer) represses translation of a target mRNA (e.g. *amiE* mRNA) by binding to A-rich sequences in the vicinity of its ribosome binding site (rbs). Under conditions when the abundance of CrcZ (in purple) increases, CrcZ binds to and titrates Hfq, which enables translation of the target genes.

CrcZ steady state levels are elevated in anoxic biofilms

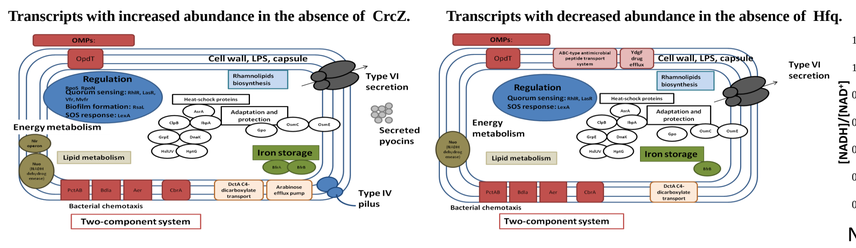


CrcZ steady state levels are elevated in anoxic biofilms grown in synthetic CF sputum medium (SCFM) as revealed by Northern blot analysis. Total RNA was extracted from planktonically growing cells (P) and from anoxic biofilms (A).

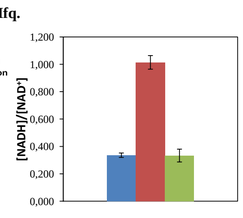
Biofilm formation is altered in *P. aeruginosa hfq*- and *crcZ*- strain due to the effect on anaerobic metabolism



Distribution of live and death cells in 96h-old anoxic biofilms of PA14, PA1Δ*crcZ* and PA14Δ*hfq* (vertical cross-section) grown in SCFM visualized by CLSM. Live cells (green) were stained with Syto 9 fluorescent dye, whereas dead cells (red) were visualized with propidium iodide.

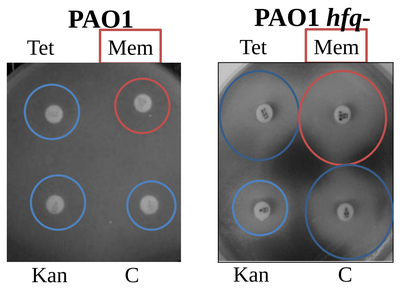


Differentially abundant genes in PA14Δ*crcZ* (left) and PA14Δ*hfq* (right) in anoxic biofilms after growth for 96h. Among them, the *nuo* operon (depicted in brown) encodes subunits of the NADH deydrogenase complex, which is required for anaerobic growth in the presence of nitrate.

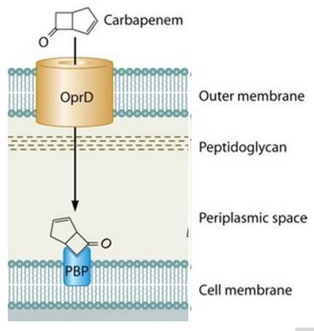


NADH/NAD+ ratio is increased in PA14Δ*hfq* in anoxic biofilms compared to PA14 indicating deregulation of *nuo* operon in PA14Δ*hfq* .

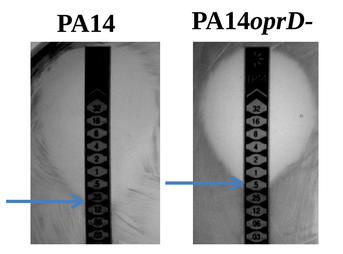
The *P. aeruginosa hfq*- strain is more susceptible to certain antibiotics



The susceptibility of *P. aeruginosa* PAO1 and the PAO1*hfq*- mutant strain towards different antibiotics was tested with a disc diffusion assay. The tested antibiotics are denoted as: tetracycline (Tet), meropenem (Mem), kanamycin (K) and chloramphenicol (C).



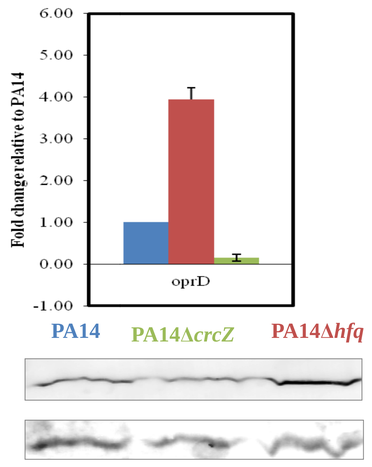
The outer membrane porin protein D (OprD) serves as a carbapenem (e.g. meropenem and imipenem) uptake channel.



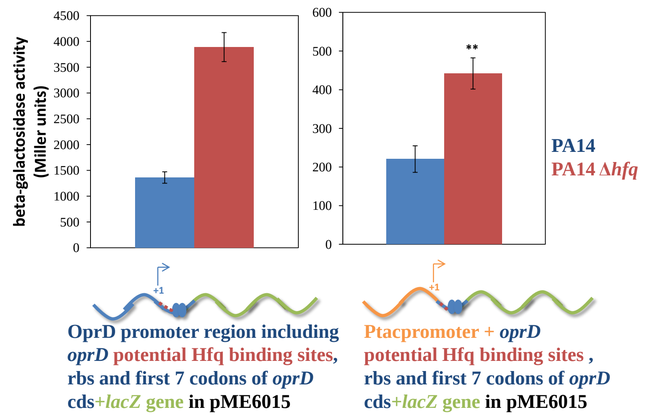
Strains	imipenem	meropenem
PA14	1	0,25
PA14*oprD*-	2	0,5

The susceptibility towards imipenem and meropenem of *P. aeruginosa* PA14 and the PA14*oprD*- mutant strain in anoxic biofilms in grown in SCFM

Negative regulation of the outer membrane porin OprD by Hfq

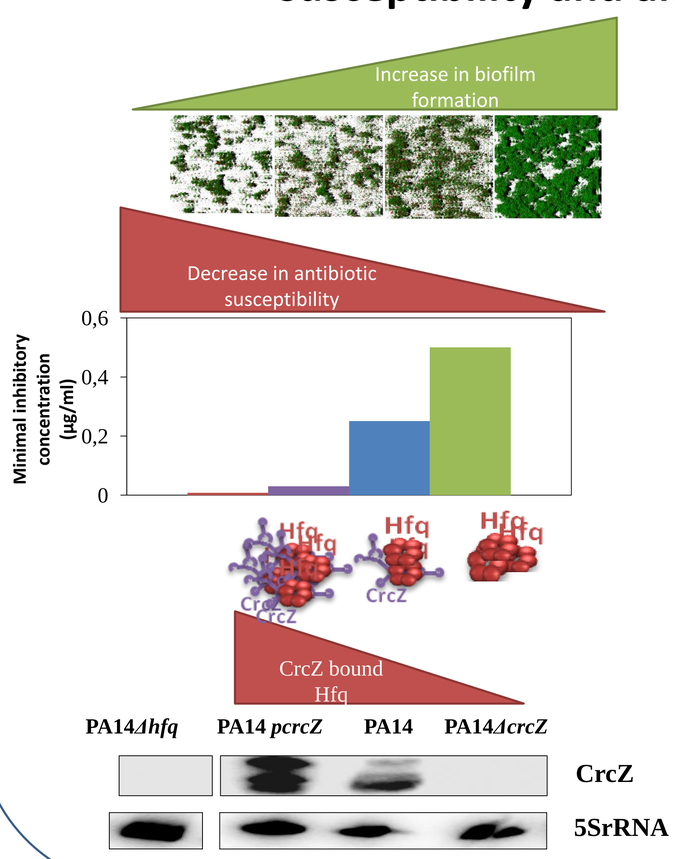


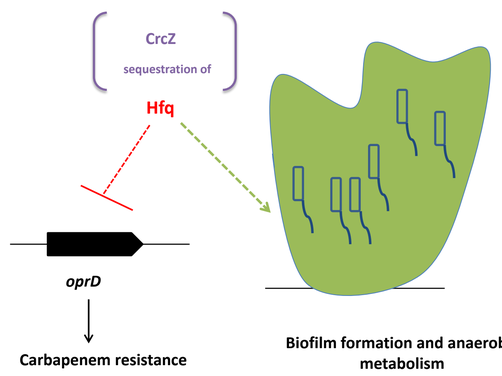
The abundance of the *oprD* transcript and the OprD protein levels were >4-fold increased in PA14Δ*hfq* (red) and 2-fold decreased in PA14Δ*crcZ* (green) when compared with PA14 (blue) in anoxic biofilms as revealed by qPCR (top), and Western blot analyses (bottom).



Hfq represses *oprD* translation. Translation of *oprD* was monitored in strains PA14 (blue) and PA14Δ*hfq* (red) grown in SCFM using translational *lacZ* reporter gene fusions. The *oprD* translational fusion constructs are schematically depicted. +1,transcriptional start sites; blue ovals, ribosome binding site; red dots: potential Hfq binding site.

Impact of CrcZ on Hfq mediated regulation of carbapenem susceptibility and anoxic biofilm formation





Model for CrcZ-Hfq mediated regulation of biofilm formation and susceptibility to carbapenems. Hfq negatively regulates expression of *oprD* leading to decreased susceptibility to carbapenems (e.g. meropenem). CrcZ counteracts Hfq-mediated regulation of *oprD* by sequestering Hfq. Hence, both the *hfq*- strain and the *crcZ* overexpressing strain show the highest sensitivity towards meropenem, whereas the *crcZ* deletion strain shows the highest resistance. At the same time Hfq promotes biofilm formation by direct/indirect effects on genes involved in anaerobic metabolism. CrcZ acts as a modulator of biofilm formation by acting as a Hfq decoy.

Conclusions

- Hfq affects anaerobic biofilm formation in *Pseudomonas aeruginosa* which can be reconciled with a direct/indirect effect of Hfq on genes required for anaerobic metabolism (e.g. *nuo* genes).
- CrcZ impacts on anaerobic biofilm formation through Hfq sequestration.
- The outer membrane porin protein D (OprD) required for carbapenem uptake is negatively regulated by Hfq.
- CrcZ counteracts the negative regulation by Hfq on *oprD* by acting as a decoy. Hence, both the *hfq*- strain and the *crcZ* overexpressing strain show the highest sensitivity towards carbapenems, whereas the *crcZ* deletion strain shows the highest resistance.

Acknowledgements

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