Highlight

Who's the boss here? The post-transcriptional global regulator Hfq takes over control of secondary metabolite production in the nematode symbiont *Photorhabdus luminiscens*.

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Comment on "*Photorhabdus*-nematode symbiosis is dependent on *hfq*-mediated regulation of secondary metabolites", by Tobias et al (2016), doi: 10.1111/1462-2920.13502

A Trojan? Dr. Jekyll and Mr. Hyde? This is *Photorhabdus luminiscens*: an enterobacterial symbiont of nematodes of the *Heterorhabditis* genus, and, at the same time, an insect killer. It colonizes the nematode gut; the worm gets into a host insect, where it regurgitates the bacteria; *P. luminiscens* then spreads throughout the insect hemolymph and secrete toxins that kill the host (Clarke, 2014). At this stage, the capacity of *P. luminiscens* to produce a wide set of secondary metabolites (SM) is turned on to serve a dual task: to keep the decaying insect tissue free of other competitor bacteria, and to serve as food and source of developmental factors for the nematode transition from infective juveniles into hermaphrodites with reproductive ability (Joyce et al., 2011) (Fig. 1). It is therefore a journey with changing environments for *P. luminiscens*, which has attracted interest not only in terms of the regulatory processes modulating its adaptation to the varying ecological niches to which it is exposed, but also because of the biological properties and diversity of the SM that it can produce. Such prolific secondary metabolism is mainly determined by a number of non-ribosomal peptide synthetase and polyketide synthase gene clusters, whose expression regulatory details have been poorly explored (Joyce et al., 2011).

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So, what was known about regulation of *P. luminiscens* behaviour before the work of Tobias and colleagues? In the insect-pathogenic state, the virulence of *P. luminiscens* requires RpoS (Qiu et al., 2016), ferric iron uptake (Watson et al., 2010), vitamin B6 (Sato et al., 2016), expression of genes for utilization of insect derived nutrients (like ethanolamine, histidine and tagatose) and of *tccC1* encoding an insecticidal toxin (Munch et al., 2008). In its symbiotic state, the catalogue comprises enzymes of the TCA cycle (Lango and Clarke, 2010) and the alarmone ppGpp (Bager et al., 2016) for a proper production of SM (particularly, stilbene which is relevant to outcompete other microorganisms (Hu and Webster, 2000)), the product of the *asmA* and *proQ* genes (which will reappear later in this text) (Easom et al., 2010), and a couple of genes encoding LysR-type transcriptional regulator proteins: HdfR (Easom and Clarke, 2012) and HexA (Joyce and Clarke, 2003). HexA has been previously reported as a repressor of several clusters for SM production (Kontnik et al., 2010) (Fig. 1). Therefore, up to now, there is little overlap in the repertoire of genetic determinants of both *P. luminiscens* symbiotic and pathogenic states (Clarke, 2014), and the sole common factor that yet appears to be important for both is the outer membrane LPS (Easom et al., 2010). So far, the identified regulatory molecules (ppGPP) and proteins (RpoS, HdfR and HexA) are part of the transcriptional layer of gene expression (Fig. 1). In addition, a singular transcriptional switch is highly relevant to modulate the transition of *P. luminiscens* cells from the pathogenic form (P) to the mutualistic and SM-producing form (M); this is a stochastic and integrase-mediated inversion of a 257-bp DNA segment containing a promoter just upstream a fimbrial operon, absolutely required for adherence and colonization of the nematode gut (Somvanshi et al., 2012).

But, what comes after transcriptional control? A master player widely –but not ubiquitously- distributed in prokaryotes is Hfq, a global post-transcriptional regulatory protein acting on diverse functional types of RNA molecules, and capable of mediating RNA-RNA interactions that in some cases bring about the RNAse degradation machinery into the game (Sobrero and Valverde, 2012; Becker, 2016). Hfq, an Sm-like protein with an hexameric toroid structure, offers alternative binding sites (its distal and proximal faces,

its rim and its outward projecting C-terminal tails) for RNA molecules (Updegrove et al., 2016). Thus, one of the broadly stablished roles of Hfq is to promote antisense and imperfect base pairing of an mRNA and a small non-coding regulatory RNA (sRNA), upon binding of both molecules to a single Hfq hexamer (Fig. 1). As a consequence, if the sRNA targets the ribosome binding site of the mRNA, there will be a translational repression, and in some cases, the RNA duplex may be subject to degradation by ribonucleases. This is the mechanistic basis of translational control for a potentially high number of mRNAs by their cognate regulatory sRNAs, which will all depend on Hfq as a common platform, thus turning Hfq into a global regulator at the post-transcriptional level (Vogel and Luisi, 2011) (Fig. 1). In fact, this has been verified in a fairly good number of bacterial species, for which mutating the *hfq* gene results in multiple phenotypic changes and the concomitant broad impact in their transcriptome and proteomes, as a result of the loss of control over multiple RNA-RNA pairs (Sobrero and Valverde, 2012).

With all this in mind, the work by Tobias and colleagues aimed to address the role of Hfq in the life cycle of *P. luminiscens*. Because in γ-proteobacteria *hfq* is part of a superoperon (Sobrero and Valverde, 2012), the authors have chosen the in-frame deletion as the correct strategy for *hfq* mutant construction. As expected, the Δ*hfq* strain showed a marked reduced yield in rich medium, and as expected, the genetic complementation of the mutant with a multicopy plasmid bearing a wild type *hfq* allele was not effective in restoring all tested phenotypes; this is also consistent with several other reports in Δ*hfq* mutants from other bacteria because altering Hfq cellular levels above the physiological steady state has an unpredictable effect on a myriad of RNA transactions. The main findings of Tobias *et al* were: 1) that the lack of Hfq impaired production of many of the SM *in vitro*, thus revealing a global regulation of SM synthesis at the post-transcriptional level; 2) the Δ*hfq* mutant is still able to kill *Galleria mellonella* larvae, but it is absolutely impaired in promoting development of *Heterorhabdis* infective juveniles into hermaphrodites, thus reinforcing the role of SM for colonization and survival within the insect tissues but not for virulence; 3) the transcriptome of the Δ*hfq* mutant revealed that the *hexA* mRNA is strongly upregulated (*ca*. 60-fold), evidencing a critical role of Hfq to

limit accumulation of *hexA* mRNA in *P. luminiscens*; 4) a double Δ*hfq* Δ*hexA* mutant recovered production of most SM, consistently with both the epistatic role of Hfq on HexA and the repressor function of HexA over SM production; 5) many other misregulated transcripts, which may be partly due to sRNA-mediated RNAse decay in the absence of Hfq or enhanced translation-coupled stability of an mRNA (post-transcriptional direct consequences), or due to the overproduction of HexA (a transcriptional indirect consequence).

One of the most relevant findings of this work is the fact that the global RNA player Hfq imposes a post-transcriptional control over the transcriptional repressor of SM HexA. A control over a control. The impact of the Δ*hfq* mutation the SM production profile implies that the expression of the biosynthetic clusters is not only coordinated at the transcriptional level by HexA (Kontnik et al., 2010) and other regulators like HdfR and TyrR (Easom and Clarke, 2012; Lango-Scholey et al., 2013), but notably, that there is a tight fine-tuning at a post-transcriptional layer that is mastered by Hfq and that may involve one or more sRNAs yet to be discovered. This post-transcriptional mastering of SM production in *P. luminescens* resembles the tight activation of a group of SM genes and operons by the post-transcriptional Gac/Rsm pathway that serve the rhizospheric biocontrol species *Pseudomonas protegens* to protect plant roots from phytopathogenic fungi and to escape predation by bacterivorous eukaryotes (Jousset et al., 2006; Lapouge et al., 2008). Overall, the results show that the three-some interaction between *P. luminiscens*, the symbiotic nematode host and the insect prey, is strongly dependent on a single protein: this is the force of one RNA binding protein.

A very important (an always desirable) consequence of a study like this is the opening of new avenues of further work. Several questions pop up. Which is the molecular mechanism laying beneath the control of Hfq over HexA? Which are the involved factors? A plausible scenario is that Hfq acts through a sRNA that affects *hexA* expression; binding of the sRNA with the assistance of Hfq often influences the stability of the sRNA target mRNAs (De Lay et al., 2013), so it would be required to compare the *hexA* mRNA half-life in the wild type and the Δ*hfq* mutant by Northern blot analysis. The

authors explored the genome of *P. luminscens* in search of a hypothetical sRNA gene whose expression may explain how the Hfq-dependent repression works over the *hexA* mRNA, and found a potential candidate that requires experimental confirmation of the existence of such putative *hexA* mRNA binding sRNA. An alternative and global approach that may also reveal the hypothetical *hexA*-sRNA as well as several other regulatory RNA pairs strongly dependent on Hfq is the application of the recently RIL-seq technology which has been proven useful to identify true *in vivo* sRNA-target interactions (Melamed et al., 2016).

Yet there would be another twist in the post-transcriptional network of *P. luminiscens*. Several years ago, a random mutagenesis approach revealed that the product of the *proQ* gene is important for the development of the nematode host (Easom et al., 2010). In that study, the authors suggested that ProQ would have a role other than the one firstly described in *E. coli*, i.e., to control levels of the ProP transport; however there is no ProP ortholog in *P. luminiscens*. The point is that it has been very recently revealed that ProQ is actually a member of the broadly conserved ProQ/FinO protein family that, in a way similar to that of Hfq, assists trans-acting sRNAs to control translation of target mRNAs (Attaiech et al., 2016; Smirnov et al., 2016). In the context of this work, the requirement of ProQ for development of *Heterorhabdis* worms is to be resignified as it may represent a parallel and complementary post-transcriptional regulatory network in *P. luminiscens*. Finally, the work by Tobias *et al*. opens yet another avenue of future work in the field of natural bioactive products, provided that derivative mutants affected in control of SM production (like the reported Δ*hfq* Δ*hexA* strain), may be a source of novel SM or of higher levels for their production *in vitro*.

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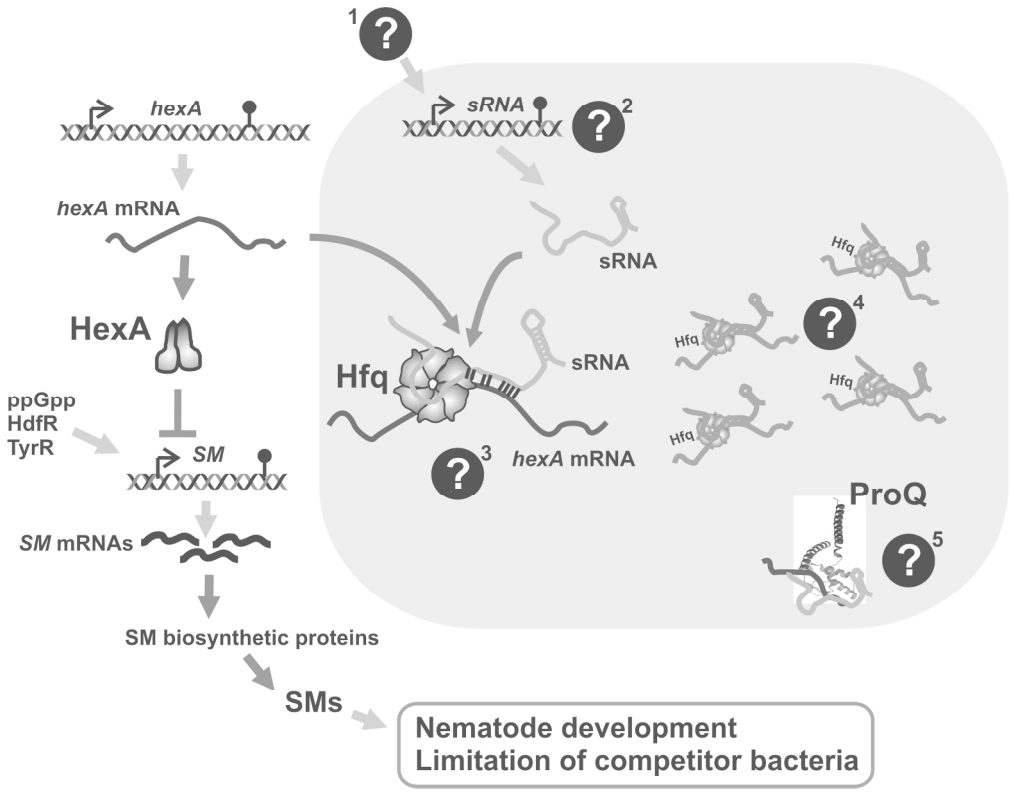
Legend to Figures

Figure 1. Interplay between the posttranscriptional (Hfq-dependent) and transcriptional (HexA-dependent) control of secondary metabolite (SM) production in *Photorhabdus luminiscens*. The regulatory model corresponds to the symbiotic state of *P. luminiscens* colonizing nematodes of the *Heterorhabdis* genus. The expression of genes encoding SM

biosynthetic proteins is fundamental for development of nematodes into reproductive hermaphrodites. Transcription of SM gene clusters requires ppGpp and the regulatory proteins HdfR and TyrR, but is repressed by the HexA regulator. Tobias *et al*. provide convincing evidences that a post-transcriptional layer of regulation takes place over the expression of SM gene clusters (gray shaded box in the Figure), and that this layer is mastered by the RNA binding protein Hfq to negatively control the level of *hexA* mRNA. Open questions in the posttranscriptional arm of the model are: the physiological stimulus (1) that triggers expression of a putative regulatory base-pairing sRNA (2) and its mechanism of downregulation of *hexA* mRNA level with the participation of Hfq (3); the occurrence of additional sRNA-mRNA regulatory pairs assisted by Hfq (4) or the recently uncovered RNA chaperone ProQ (5).



Interplay between the posttranscriptional (Hfq-dependent) and transcriptional (HexA-dependent) control of secondary metabolite (SM) production in Photorhabdus luminiscens. The regulatory model corresponds to the symbiotic state of P. luminiscens colonizing nematodes of the Heterorhabdis genus. The expression of genes encoding SM biosynthetic proteins is fundamental for development of nematodes into reproductive hermaphrodites. Transcription of SM gene clusters requires ppGpp and the regulatory proteins HdfR and TyrR, but is repressed by the HexA regulator. Tobias et al. provide convincing evidences that a post-transcriptional layer of regulation takes place over the expression of SM gene clusters (gray shaded box in the Figure), and that this layer is mastered by the RNA binding protein Hfq to negatively control the level of hexA mRNA. Open questions in the posttranscriptional arm of the model are: the physiological stimulus (1) that triggers expression of a putative regulatory base-pairing sRNA (2) and its mechanism of downregulation of hexA mRNA level with the participation of Hfq (3); the occurrence of additional sRNA-mRNA regulatory pairs assisted by Hfq (4) or the recently uncovered RNA chaperone ProQ (5).
Fig. 1