

The Regulation of Secondary Metabolism in *Photorhabdus*

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Abstract A general definition of secondary metabolism is that it consists of the metabolic pathways and the products of metabolism that are not absolutely required for the survival of the organism. Using this definition, it is now well established that *Photorhabdus* elaborate an extensive secondary metabolism during the post-exponential phase of bacterial growth. This secondary metabolism includes, but is not limited to, the production of light, a stilbene antibiotic and an anthraquinone pigment. In this chapter, the role of secondary metabolism during the life cycle of *Photorhabdus* will be discussed. Recent work has shown that secondary metabolism in *Photorhabdus* is required for the mutualistic association between the bacteria and its nematode partner, in particular bacterial secondary metabolism is required to support normal nematode growth and development. An isogenic population of *Photorhabdus* is phenotypically heterogenous and this facilitates functional partitioning within the population. The relationship between secondary metabolism and the various phenotypic and phase variants that exist in populations of *Photorhabdus* will also be discussed. Finally, this chapter will also describe the various regulatory nodes that have been identified as being part of the complex regulatory network that is used to control the temporal expression of secondary metabolism in *Photorhabdus*.

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1 Introduction

Secondary metabolism can be defined as consisting of the metabolic pathways and the products of metabolism that are not absolutely required for the survival of the organism. Many bacteria, e.g. *Streptomyces*, have important secondary metabolisms that are responsible for the production of a range of clinically important molecules, such as antibiotics and immunosuppressive drugs. Secondary metabolism is generally associated with the post-exponential or stationary phase of bacterial growth when nutrients are limited and metabolic flux is redirected away from the production of new bacterial cells (i.e. primary metabolism) to the production of secreted metabolites (i.e. secondary metabolism). However, there is no reason why secondary metabolites cannot also be produced during bacterial growth and it is now clear that secondary metabolism in bacteria is regulated by a complex network of global and pathway-specific regulators that link primary and secondary metabolism. The role of secondary metabolism (and the function of the resulting secondary metabolites) during the normal life of a bacterium is not fully understood but these molecules may act as signals to modulate the interaction between the producing bacterium and other organisms in the ecosystem (Romero et al. 2011).

Members of the *Photorhabdus* genus have an extensive secondary metabolism that includes the production of a wide range of different activities during the stationary phase of bacterial growth (Clarke 2008; Waterfield et al. 2009; Bode 2009). These activities include the genus defining characteristic of bioluminescence (i.e. light production) as well as the production of a number of small bioactive molecules such as a multipotent stilbene and a polyketide pigment called anthraquinone (AQ). The metabolic pathways responsible for the production of both stilbene and AQ have been described and will be discussed elsewhere in this book (see Chap. 20) (Brachmann et al. 2007; Joyce et al. 2008). Many secondary metabolites are produced through the action of enzymes called polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS) and recent work has identified a significant number of genetic loci encoding potential PKS, NRPS and PKS-NRPS chimeras on the *Photorhabdus* genome (Bode 2009). Many of these loci are cryptic and therefore the genes are not expressed under normal laboratory conditions (Brachmann et al. 2012a). This suggests that the expression of these loci is tightly regulated in *Photorhabdus*. Nonetheless, a range of biochemical and genetic approaches have been used to identify and characterize a significant number of the molecules produced by the enzymes encoded at several of these loci (see Table 1 and references therein).

Table 1 Secondary metabolites produced by *Photorhabdus luminescens* TTO1

Metabolite	Genetic loci	Role	References
Stilbene	<i>stlA; stlB; stlCDE; bkdABC</i>	Antibiotic; IJ recovery signal; PPO inhibitor	Williams et al. (2005) and Joyce et al. (2008)
Pyrone ^b	<i>ppyS</i>	Quorum sensing	Brachmann et al. (2013)
Lumiquinone A	<i>plu2233?</i>	Unknown	Park and Crawford (2015)
Dihydrophenylalanine (dihydrostilbene)	<i>plu3042-3044</i>	Unknown	Crawford et al. (2011)
Anthraquinone	<i>antABCDEFGHI</i>	Redox active; possible mosquitocidal activity	Brachmann et al. (2007) and Ahn et al. (2013)
Glidobactin/Cepafungin I	<i>plu1877-1881</i>	Proteasome inhibitors	Bian et al. (2012), Theodore et al. (2012), Dudnik et al. (2013) and Stein et al. (2012)
Rhabduscin	<i>plu1760-1762; insA-insB</i>	PPO inhibitor	Crawford et al. (2012)
Phurealipids	<i>pliA</i>	Juvenile hormone epoxide hydrolase inhibitor	Nollmann et al. (2015)
Indigoidine ^a	<i>plu2180-2185-indC-indA</i>	Blue pigment	Brachmann et al. (2012a)
GameXpeptides/luminmides	<i>plu3263; plu3561-3567</i>	Unknown	Bode et al. (2012) and Fu et al. (2012)
Kollisin A ^a	<i>kol (plu2670)</i>	Unknown	Bode et al. (2015)

^aCryptic^b*P. asymbiotica* use dialkylresorcinol instead of pyrone as a QS signalling molecule (synthesized by *darABC* [same as *stlCDE* in *P. luminescens* TTO1])

2 The *Photorhabdus* Life Cycle

Photorhabdus has a complex life cycle that involves alternating and temporally distinct pathogenic and mutualistic interactions with insect and nematode hosts, respectively (Goodrich-Blair and Clarke 2007; Waterfield et al. 2009; Clarke 2014). The bacteria are normally found in the gut lumen of the infective juvenile (IJ) of the soil-dwelling nematode *Heterorhabditis*. The IJ is a specialized developmental stage of the nematode, similar to the dauer juvenile of the well-studied free living nematode *Caenorhabditis elegans*. The IJ actively seeks out and infects suitable insect larvae before releasing the bacteria into the blood (i.e. hemolymph) of the

infected insect. The regurgitation of the bacteria is a response to a small, unidentified signal present in the insect hemolymph and regurgitation signals the start of a developmental pathway, called IJ recovery, that results in the formation of a self-fertile adult hermaphrodite from every IJ (Ciche and Ensign 2003). The bacteria replicate in the insect and, whilst doing so, convert the internal organs and tissues of the insect into bacterial biomass. The insects generally die within 48–72 h post-infection. Bacterial growth in the insect is exponential and there is a very strong positive correlation between bacterial growth rate and the time taken to kill an insect (generally represented as an LT_{50}) suggesting that virulence is associated with bacterial growth and, therefore, primary metabolism (Clarke and Dowds 1995; Watson et al. 2005). The bacteria replicate to a high cell density within the insect and, as in any batch system, the bacterial population eventually enter the post-exponential (or stationary) phase of growth. This generally coincides with the completion of IJ recovery. At this time, the adult hermaphrodite nematodes are present in the insect cadaver with a high density of *Photorhabdus* bacteria cells (Hu and Webster 2000). The hermaphrodite will externally lay about 300 eggs that hatch within the bacterial biomass present in the insect cadaver and develop through several juvenile moults (J1–J4) to adulthood. Normally, there are 2–3 generations of nematode reproduction in the insect and during this time the nematodes feed on the bacterial biomass. The adult hermaphrodite does not lay all of her eggs and the retained eggs will hatch within the body cavity of the maternal nematode in a process called *endotokia matricida* (Ciche et al. 2008). The internally hatched nematodes develop exclusively into the IJ stage and, during development in the hermaphrodite, each IJ is colonized by *Photorhabdus*. The colonization of the IJs (i.e. symbiont transmission) is controlled by a phase variable invertible DNA switch in *Photorhabdus* called the *madswitch* (Somvanshi et al. 2010, 2012). The *madswitch* is a binary switch that can exist in one of 2 orientations, i.e. ON or OFF. When the *madswitch* is ON, the bacteria produce surface-localized fimbriae, called the Mad fimbriae, that facilitate adherence to the intestinal wall of the maternal nematode and subsequent colonization of the IJ. Each IJ is normally initially colonized by 1 bacterial cell and this bacterial cell multiplies in the gut lumen of the nematode such that mature IJs contain, on average, approximately 100 bacterial cells (Somvanshi et al. 2010). The complete life cycle normally takes 10–20 days and >100,000 IJs will emerge from an insect originally infected with a single IJ highlighting the remarkable efficiency of this symbiosis.

3 Secondary Metabolism, Pathogenicity and Mutualism

The mutualistic interaction between *Photorhabdus* and *Heterorhabditis* coincides with the post-exponential phase of bacterial growth in the insect (Hu and Webster 2000). Therefore, mutualism also coincides with the activation of *Photorhabdus* secondary metabolism, thus temporally connecting secondary metabolism and mutualism (Joyce et al. 2011). What is the role of secondary metabolism in

mutualism? Perhaps the most significant evidence for a role for secondary metabolism in mutualism was extrapolated from early observations of phenotypic variation in *Photorhabdus* (Akhurst 1980; Boemare and Akhurst 1988). *Photorhabdus* can exist as 2 stable phenotypic variants when cultured on agar plates, i.e. the primary and the secondary variants. The primary variant has a normal secondary metabolism (including the production of AQ, stilbene and light) and is both pathogenic to insects and capable of supporting nematode growth and development. The secondary variant, on the other hand, does not produce AQ, stilbene or light and, whilst this variant is pathogenic, it is unable to support nematode growth and development. Therefore, it has been proposed that secondary metabolism (as characterized in the primary variant) is important for the mutualistic association with the nematode (Ffrench-Constant et al. 2003). However, only one secondary metabolite has been shown to have a direct role in mutualism, i.e. the stilbene antibiotic. During in vitro symbiosis assays (where the *Photorhabdus* bacteria are inoculated onto lipid agar plates and incubated for 3–4 days before the biomass is seeded with IJ stage nematodes), mutants unable to produce stilbene do not support IJ recovery to adult hermaphrodites (Williams et al. 2005; Joyce et al. 2008). This suggests that the stilbene is an inter-kingdom signalling molecule that is produced by the bacteria and is perceived by the IJ nematode and signals initiation of the recovery programme. However, during growth in the insect, the role of the stilbene may be to act as a food signal that stimulates the recovery of IJs that develop during the normal reproductive cycle of the nematodes (Strauch and Ehlers 1998). This would function to link nematode reproduction and development with the availability of food, i.e. *Photorhabdus* biomass. The primary variant of *Photorhabdus* also produces 2 crystalline inclusion proteins that are rich in essential amino acids during post-exponential growth leading to the suggestion that these proteins provide important nutrition to the nematode (Bowen and Ensign 2001; You et al. 2006). However, in vitro studies suggest that, with the exception of stilbene, the other activities associated with secondary metabolism in *Photorhabdus* may not be required for nematode growth and development (Han and Ehlers 2001; Bager et al. 2016). The bottleneck for nematode growth and development when cultured on lipid agar plates inoculated with *Photorhabdus* appears to be IJ recovery, a process that during in vitro symbiosis assays is almost entirely dependent on the production of stilbene. Therefore, mutants that do not express the normal secondary metabolism exhibit a decrease in nematode growth and development due to the reduced production of stilbene and lower levels of IJ recovery (Bager et al. 2016). Nonetheless, it is possible that many activities associated with secondary metabolism in *Photorhabdus* may have non-nutrition-based roles in the interaction with the nematode during growth in the insect, e.g. the production of signals that protect the insect cadaver niche (Fenton et al. 2011; Jones et al. 2016).

Whilst secondary metabolism is normally associated with the post-exponential phase of bacterial growth, there is evidence that some secondary metabolites are produced during exponential phase (i.e. during bacterial growth). The growth rate of *Photorhabdus* has been correlated with virulence suggesting that any secondary metabolite produced during this phase of growth may have a role in virulence. The

expression of the *cpm* genes encoding the proteins required for the production of the carbapenem antibiotic by *Photorhabdus* is maximal during exponential growth and decreases as the cells enter post-exponential growth (Derzelle et al. 2002). Whilst unlikely to be a virulence factor *per se*, the production of this antibiotic would be expected to increase the fitness of *Photorhabdus* by killing off the indigenous insect microbiota. In addition, biochemical and genetic analyses have identified a very potent antagonist of prophenoloxidase (PPO), called rhabduscin, that is produced by *Photorhabdus*. PPO is a key enzyme in the insect innate immune system that catalyses the polymerization of melanin resulting in the death and/or nodulation/encapsulation of invading organisms (Cerenius and Söderhäll 2004; Kanost et al. 2004; Cerenius et al. 2008). A deletion of the genes required for rhabduscin biosynthesis in the closely related entomopathogenic bacterium *Xenorhabdus* did result in a decrease in virulence suggesting that this compound may be a virulence factor. The rhabduscin was shown to be physically associated with the outer surface of the bacteria resulting in the formation of a “defensive perimeter” (Crawford et al. 2012). Rhabduscin has been detected in *P. luminescens* TTO1 culture supernatants although the kinetics of rhabduscin production has not been measured (Crawford et al. 2010).

4 Phenotypic Variation, Phase Variation and Secondary Metabolism

As previously mentioned (nearly), every IJ is initially colonized by a single bacterium and, therefore, an infected insect is expected to contain a clonal population of *Photorhabdus*. However, there is a significant degree of functional specialization within this clonal population. It has already been mentioned that *Photorhabdus* can exist in 2 relatively stable phenotypic variants when cultured on agar plates, i.e. the primary and secondary variants. It is now well established that many bacteria exhibit phenotypic variation (i.e. bistability) as a risk-limiting adaptation that increases the adaptability of a population of isogenic bacteria to changing environments (Dubnau and Losick 2006; Veening et al. 2008; Ackermann 2015). This survival strategy when displayed by isogenic populations is called bet-hedging. The rate of formation of the secondary variant has been shown to increase as the population of *Photorhabdus* gets older (e.g. over timescales that are similar to the time required for nematode growth and development) and the kinetics of phenotypic variation is controlled by the AstSR two-component pathway (2CP) (Derzelle et al. 2004). Mutants in the *astR* gene (encoding the predicted response regulator) exhibit increased and promiscuous (i.e. early) formation of the secondary variant, and proteomic analysis of this mutant suggests that this phenotype may be linked to defects in redox homeostasis. Therefore, the AstSR 2CP may control phenotypic variation in response to nutrient limitation and redox stress (Derzelle et al. 2004). Interestingly, the AstSR 2CP does not regulate secondary metabolism directly

(i.e. the *astR* mutant produces the same level of light, stilbene and AQ as the wild type) but this pathway can obviously control (through phenotypic variation) the level of secondary metabolism within a population of *Photorhabdus*.

The vast majority of *Photorhabdus* inside an insect will not have the opportunity to colonize the IJ and these bacteria will remain in the insect cadaver after the nematodes have left (see Fig. 1). As secondary metabolism is associated with life with the nematode, the switch from primary variant to secondary variant (with the associated switching off of secondary metabolism) may be an adaptation for life in the environment without the nematode. This hypothesis is supported by comparisons of the metabolism and proteomes of primary and secondary variants that suggest that the secondary variant is metabolically more robust than the primary variant during growth in vitro and in vivo (Smigielski et al. 1994; Turlin et al. 2006) (Susan Joyce, unpublished data).

In addition to phenotypic variation, *Photorhabdus* has also been shown to exhibit phase variation, mediated by invertible DNA elements, at 2 different genetic loci, i.e. the *mrf* operon (encoding mannose-resistant fimbriae) and the *mad* operon (encoding the Mad fimbriae) (Meslet-Cladiere et al. 2004; Somvanshi et al. 2010). As already mentioned, the production of the Mad fimbriae is controlled by the orientation of a DNA element called the *madswitch*. However, the *madswitch* also controls the production of a number of additional phenotypes, including secondary metabolism (Somvanshi et al. 2012). *Photorhabdus* with the *madswitch* in the OFF orientation are phenotypically equivalent to the primary variant (hereafter called the P-form). The P-form has a normal secondary metabolism and is both pathogenic to

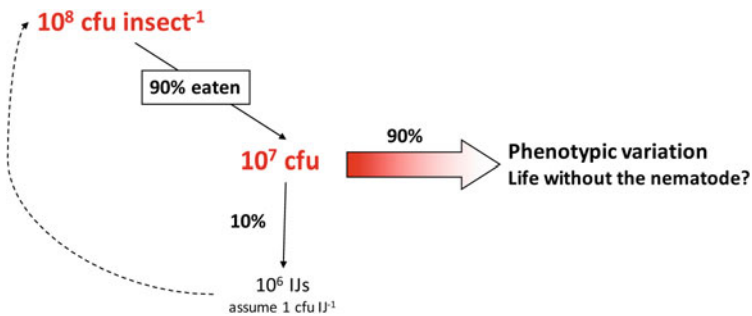


Fig. 1 Secondary variant is adapted for life without the nematode. *Photorhabdus* achieve a final cell density of approximately 10^8 bacterial cells [colony forming units (cfu)] per insect. It is not unreasonable to assume that 90 % of the bacterial biomass will be eaten by the nematodes that are developing within the insect cadaver. This scenario would suggest that 10^7 cfu could remain in the insect at the end of the vegetative life cycle of the nematode. Assuming that each insect supports the development of 10^6 IJs and each IJ is colonized by a single *Photorhabdus* cell then a very conservative estimation is that 9×10^6 cfu remain in the insect cadaver after the nematode has left. A simplistic model would suggest that these bacteria are converted to the secondary variation in order to adapt to life without the nematode. However, it is more likely that secondary variants will accumulate during growth of the bacteria in the insect (a phenomenon regulated by HexA and AstSR) and the proposed model suggests that the system is buffered so that phenotypic variation is tolerated, up to a point, without deleterious consequences to nematode growth

insects and able to support nematode growth and development. However, the P-form is not able to colonize the IJ as it is not producing the Mad fimbriae. On the other hand, *Photorhabdus* with the *madswitch* in the ON orientation (i.e. M-form) are able to colonize the IJ. However, the M-form bears some phenotypic resemblance to the secondary variant, i.e. the M-form does not produce light, the stilbene antibiotic or the AQ pigment. Therefore, a mixture of P-form and M-form bacteria is essential for the complete mutualistic association with the nematode. At some point after the colonization of the adult hermaphrodite, it has been shown that the M-form reverts back to the P-form (Somvanshi et al. 2010). This ensures that the IJ is colonized by the P-form so that the nematodes will have the required nutrition following infection of a new insect host. In addition, there is some evidence to suggest that the formation of the M-form, like the secondary variant, is linked to nutrient limitation and/or oxidative stress (Somvanshi et al. 2012; Blackburn et al. 2016). However, the M-form is not virulent to insects and M-form bacteria form small colonies [i.e. small colony variants (SCVs)] on agar plates compared to the P-form and secondary variant (see Fig. 2). Therefore, the M-form and the

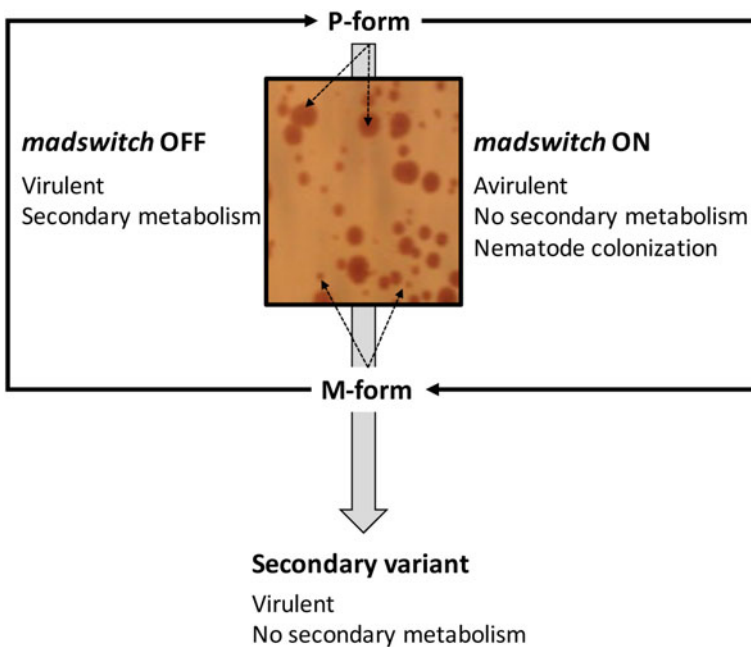


Fig. 2 Phenotypic and phase variation in *P. luminescens* TTO1. A population of *P. luminescens* exists as a mixture of P-form and M-form cells determined by the orientation of a binary DNA switch, the *madswitch*. When plated on agar, the P-form cells form large colonies whilst the M-form cells form small colonies (as indicated). The P-form is virulent and produces a secondary metabolism (i.e. supports nematode growth and development) whilst the M-form is adapted for colonization of the nematode and, therefore, it is avirulent and does not have a secondary metabolism. *Photorhabdus* also form a secondary variant but the origins of the secondary variant are unclear; i.e. is it derived from the P-form or M-form? Indeed, the secondary variant shares phenotypes with both the M-form and the P-form but these variants are distinct

secondary variant are not equivalent and the regulatory networks controlling secondary metabolism during phenotypic variation and phase variation at the *mad* locus would appear to be different.

Nonetheless, the complex phenomena of phenotypic and phase variation ensure that there is functional heterogeneity within a clonal population of *Photorhabdus*, such as exists in the insect cadaver. This enables the bacteria to carry out several distinct functions that are essential for mutualism with the nematode and the survival of the bacteria, i.e. the ability to express secondary metabolism and support nematode growth and development, the ability to colonize the next generation of IJs (i.e. transmission) and the ability to persist in the environment in the absence of the nematode.

5 The Regulation of Secondary Metabolism

It is clear that secondary metabolism is differentially expressed in the different phenotypic and phase variants of *Photorhabdus* that have been characterized to date. Studies aimed at describing the structure of the regulatory network(s) controlling secondary metabolism have been undertaken and key players (i.e. regulatory nodes) have been identified. Most of these studies have been undertaken in the primary variant where efforts have been made to understand the regulatory link between secondary metabolism and mutualism and important global and pathway-specific regulators have been identified. Some of these regulators have also been implicated in the regulation of secondary metabolism in the secondary variant and M-form. Finally, it is worth highlighting that the vast majority of these studies have been done using the de facto type strain of *Photorhabdus*, *P. luminescens* TTO1.

5.1 Global Regulators

5.1.1 HexA

A key regulator of secondary metabolism in *Photorhabdus* is a LysR-type transcriptional regulator called HexA. This regulator was initially identified as a repressor of secondary metabolism in the secondary variant. Interruption of the *hexA* gene in the secondary variant of *P. temperata* K122 restored the expression of secondary metabolism (and the ability to support nematode growth and development) to this strain (Joyce and Clarke 2003). However, HexA also appears to have a regulatory role in the primary variant where it is required for the correct temporal regulation of secondary metabolism. Therefore, a *hexA* deletion mutant in the primary variant of *P. luminescens* TTO1 produces increased levels of light, stilbene and derivatives of the stilbene molecule throughout growth and not just in the

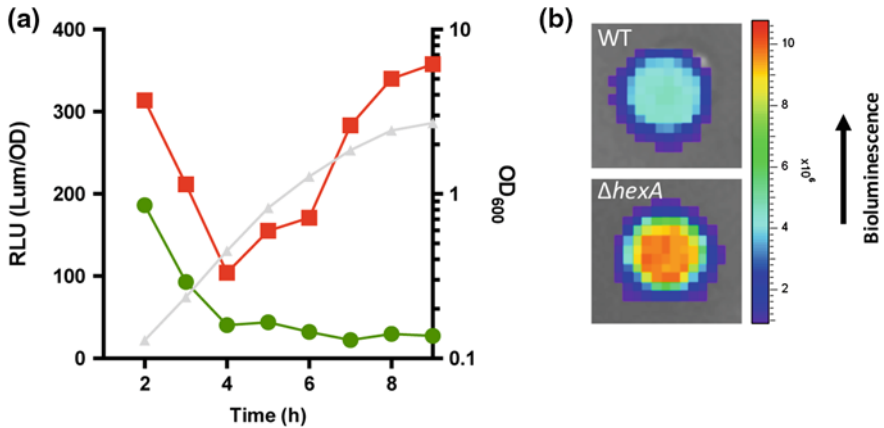


Fig. 3 HexA represses bioluminescence during growth of the *P. luminescens* TTO1 primary variant. **a** Light production (reported as Relative Light Units (RLU)) from *P. luminescens* primary variant (*green circles*) and $\Delta hexA$ mutant (*red squares*) was measured during growth in LB broth at 28 °C with shaking. There were no differences in the growth of either strain under these conditions (*grey triangles*). **b** The light produced by colonies of TTO1 primary variant and the $\Delta hexA$ mutant was measured after growth on LB agar for 72 h. Light production from the $\Delta hexA$ mutant is clearly increased compared to the primary variant control. Bioluminescence was measured using an In Vitro Imaging System (IVIS-100, Xenogen Alameda, CA, USA)

post-exponential phase (Kontnik et al. 2010) (see Fig. 3). Interestingly, HexA repressed AQ production in *P. temperata* but not in *P. luminescens* suggesting species-specific regulatory targets (Kontnik et al. 2010). HexA may also have a role in repressing secondary metabolism in the M-form as transcriptome analysis has indicated that the expression of *hexA* is increased by 4.4-fold in a strain that is genetically locked into the M-form (Somvanshi et al. 2012). Therefore, HexA appears to be an important repressor of secondary metabolism in *Photorhabdus*. However, the regulatory network(s) controlled by HexA in the primary and secondary variants (and potentially the M-form) are not well understood.

5.1.2 The BarA-UvrY Two-Component Pathway

The BarA-UvrY 2CP activates the transcription of genes involved in secondary metabolism in *Photorhabdus*. A deletion mutant in *uvrY* (encoding the response regulator) in *P. luminescens* exhibited much lower levels of expression of the genes required for bioluminescence, stilbene and AQ biosynthesis (Krin et al. 2008). However, many of the transcriptional changes observed in the *uvrY* mutant strain were not evident at either the protein or phenotypic level suggesting that the expression of these genes may also be significantly regulated at the

post-transcriptional level (Krin et al. 2008; Kontnik et al. 2010; Lango-Scholey et al. 2013). Indeed, to this end, the BarA-UvrY 2CP in other bacteria has been shown to work in association with small regulatory RNA molecules. A well-studied target of the BarA-UvrY 2CP is the CsrA-*csrB* regulatory system (Pernestig et al. 2003; Weillbacher et al. 2003). In this system, CsrA is a RNA-binding protein that represses translation by binding to the ribosome-binding site of the target mRNA (Liu and Romeo 1997). The *csrB* RNA carries multiple target sites from CsrA and, when expressed, titrates CsrA away from target mRNAs, thus increasing the translation efficiency of these transcripts (Romeo 1998; Babitzke and Romeo 2007). In *P. luminescens* TTO1, the CsrA-*csrB* regulatory system was shown to play a role in the regulation of some, but not all, of the identified UvrY targets (Krin et al. 2008). The BarA-UvrY 2CP is also known as GacS-GacA in other bacteria such as *Pseudomonas* where the GacS-GacA 2CP has also been shown to have an important role in the regulation of secondary metabolism (Lapouge et al. 2007). Similar to what has been observed in *Photorhabdus*, mutations in the GacS-GacA 2CP (a pathway that works together with the Rsm RNA regulatory system) block secondary metabolism in several different species of pseudomonads (Heeb and Haas 2001). Interestingly, recent work has also shown that *gacS* mutations in the rhizobacterium *Pseudomonas aeruginosa* M18 generate phenotypic diversification resulting in the formation of SCVs both in vivo and in vitro (Davies et al. 2007; Nelson et al. 2010). Moreover, the GacS-GacA system was shown to have an interesting role in the association between *Ps. fluorescens* and the bacterial-feeding, social amoeba *Dictyostelium discoideum*. In this system, many *D. discoideum* have been observed to engage in bacterial husbandry by carrying and disseminating the bacteria during the spore stage, thus ensuring that there is food available for the amoeba following germination of the spores. In initial studies, *D. discoideum* was shown to carry 2 strains of *Ps. fluorescens* although only 1 strain served as a food source for the amoeba (Brock et al. 2011). Recently, it was shown that the second strain produced secondary metabolites, chromene and pyrrolnitrin that were beneficial to the amoeba by enhancing appropriate spore formation. Intriguingly the secondary metabolite-producing strain could be converted into the food source by a loss-of-function mutation in the *gacA* gene (Stallforth et al. 2013). Therefore, the *gacA* mutation generates functional heterogeneity within the population of *Ps. fluorescens*. *Photorhabdus* has similar roles during the mutualistic association with the nematode (i.e. it is a food source for the nematode and it produces molecules that control nematode development). Although there is no evidence to suggest that non-functional alleles of *uvrY* (the homologue of *gacA*) emerge and co-exist within insect cadavers it is possible that the activity of the BarA-UvrY 2CP is modulated (e.g. post-transcriptionally) to induce functional heterogeneity within the bacterial population (Camacho et al. 2015). Nonetheless, it is clear that the homologous BarA-UvrY and GacS-GacA 2CPs play a key role in the regulation of functional heterogeneity and secondary metabolism in different bacteria–host interactions.

5.1.3 Magic Spot-(P)ppGpp and Nutrient Limitation

A recent study in *Phototrhobdus* showed that the expression of *stlA*, encoding phenylalanine ammonium-lyase (PAL), was regulated by the availability of nutrients, in particular amino acids (Lango-Scholey et al. 2013). PAL is the first enzyme in the synthesis of the stilbene antibiotic and this enzyme converts the amino acid phenylalanine into cinnamic acid (CA) further highlighting the connection between amino acid metabolism and secondary metabolism (Williams et al. 2005). It is well established throughout the gammaproteobacteria that nutrient limitation results in the production of a small signalling molecule (or alarmone) called (p)ppGpp (Gaca et al. 2015a; Hauryliuk et al. 2015). The production of (p)ppGpp generally results in the halting of protein synthesis and DNA replication and, through an interaction with RNA polymerase, the biasing of transcription towards genes required for adaptation to the stress. The level of (p)ppGpp in the bacterial cell is controlled by the activity of two proteins, RelA and SpoT. Recent work has shown that RelA monitors the aminoacylated-status of the tRNA present in the A site of the ribosome (Brown et al. 2016). RelA is activated when uncharged tRNA molecules enter the A-site (indicating amino acid limitation) and this results in the synthesis of (p)ppGpp. RelA appears to respond primarily to amino acid starvation whilst SpoT integrates various other signals into (p)ppGpp metabolism. Moreover, the hydrolysis activity of SpoT is essential for maintaining appropriate levels of (p)ppGpp during growth (Hauryliuk et al. 2015).

The accumulation of (p)ppGpp has been shown to result in significant transcriptional changes in many bacteria (Hesketh et al. 2007; Traxler et al. 2010; Vercruyssen et al. 2011; Bowden et al. 2013; Gaca et al. 2015b). In *E. coli*, structural and genetic analyses suggest that the small alarmone interacts directly with RNA polymerase although the exact molecular details of how this interaction affects transcription are not fully understood (Zuo et al. 2013; Ross et al. 2013). The synthesis of (p)ppGpp is often linked to nutrient limitation and growth arrest and this is likely to coincide with a change from primary to secondary metabolism. In a recent study, the accumulation of (p)ppGpp was shown to be required for secondary metabolism and mutualism in *Phototrhobdus* (Bager et al. 2016). Therefore, a *relA spoT* double mutant was constructed and shown to be completely unable to synthesize (p)ppGpp. The *relA spoT* mutant did not produce light, stilbene or AQ pigment and was also unable to support nematode growth and development in vitro. This suggests, perhaps paradoxically (given the role for *Phototrhobdus* in nematodes nutrition), that nutrient limitation in *Phototrhobdus* is important for mutualism. In contrast, the *relA spoT* mutant was as virulent to insects as the wild-type strain indicating the alarmone production was not required for pathogenicity. However, the *relA spoT* mutant was rapidly out-competed by the wild-type strain during prolonged incubation in the insect again highlighting the important role for (p)ppGpp during nutrient limitation (Bager et al. 2016). The (p)ppGpp alarmone has also been shown to be required for the production of secondary metabolites such as the blue polyketide pigment, actinorhodin and the calcium-dependent antibiotic (CDA) in the model actinomycete, *Streptomyces coelicolor* (Hesketh

et al. 2007). Moreover, a *relA spoT* mutant in *Ps. fluorescens* CHAO had decreased antibiotic activity and biocontrol activity suggesting a role for (p)ppGpp in the regulation of secondary metabolism in this bacterium (Takeuchi et al. 2012). Indeed, it seems that the level of (p)ppGpp in *Ps. fluorescens* CHAO can be regulated by the GacA-GacS 2CP system highlighting the complexity and interconnectedness of the networks involved in controlling secondary metabolism (Takeuchi et al. 2012).

5.1.4 Metabolism and the Metabolic Switch

Primary metabolism is generally associated with the central metabolic pathways (i.e. glycolysis, the TCA cycle) that produce energy and metabolic precursors to facilitate cell growth and reproduction. Secondary metabolism, on the other hand, can be considered as an alternative route for carbon and energy flux that leads to the production of carbon-rich bioactive compounds (rather than the production of bacterial biomass). Therefore, it is entirely appropriate that primary and secondary metabolism should be tightly linked, in terms of both regulatory and metabolic connections. Deletion of either the *mdh* or *fumC* gene and encoding key enzymes in the TCA cycle (malate dehydrogenase and fumarase, respectively) were shown to block the production of light, stilbene antibiotic and AQ pigment in *P. luminescens* TTO1 (Lango and Clarke 2010). As expected both the *mdh* and *fumC* mutants were also unable to support nematode growth and development in vitro or in vivo and both mutants were unaffected in virulence. This led to the suggestion that the transition from pathogenicity (i.e. *Photorhabdus* growth) to mutualism (i.e. *Photorhabdus* secondary metabolism) was controlled by a metabolic switch that involved the TCA cycle. Interestingly, a similar block in the TCA cycle in *Ps. fluorescens* CHAO (specifically a mutation in the *fumA* gene encoding fumarase) also blocked secondary metabolism in this bacterium where it was shown that an imbalance in the TCA cycle (leading to an accumulation of certain TCA cycle intermediates) prevented the appropriate activation of the GacS-GacA 2CP (Takeuchi et al. 2009). Similarly, a mutation in the *acnB* gene (encoding aconitase, a key TCA cycle enzyme) of the bioluminescent bacterium *Vibrio fischeri* resulted in increased bioluminescence and this was shown to be dependent on the Gac/Rsm regulatory system (Septer et al. 2015). Therefore, the BarA-UvrY (GacS-GacA) 2CP does appear to constitute a clear regulatory link between primary and secondary metabolism in many different bacteria.

L-proline is an abundant free amino acid in insect haemolymph and increasing concentrations of L-proline upregulate the production of the stilbene antibiotic and AQ pigment in *P. luminescens* TTO1 (Crawford et al. 2010). This phenotype was shown to be dependent on proline uptake and there is some evidence that the role of proline in secondary metabolism may be regulatory as L-proline may interact with HexA (Kontnik et al. 2010). However, L-proline assimilation also contributes to the generation of proton motive force (PMF) and dissipating the PMF with either CCCP or valinomycin resulted in increased levels of stilbene antibiotic and the AQ

pigment suggesting that the electrical component of the proton motive force is linked to secondary metabolism (Crawford et al. 2010). Therefore, the presence of L-proline may have a role in coordinating the link between the PMF and the secondary metabolism in *Phototrhobdus*.

5.1.5 Quorum Sensing and AI-2

Quorum sensing is a phenomenon in bacteria that is characterized by the production of signal molecules that mediate cell-to-cell communication and coordinates gene expression (Miller and Bassler 2001; Ng and Bassler 2009). *Phototrhobdus* do not communicate using canonical quorum-sensing signals such as acylhomoserine lactones (AHLs). However, many bacteria have also been shown to quorum sense using an alternative signal called AI-2 (Pereira et al. 2013). This signal is produced by the product of the *luxS* gene, S-ribosylhomocysteine lyase, that has an important metabolic function during the activated methyl cycle where LuxS converts S-D-ribosyl-L-homocysteine into L-homocysteine. A product of this reaction is AI-2 and this molecule is excreted from the cell where it accumulates in the extracellular environment. The production of AI-2 has been shown to induce transcriptional changes in some bacteria leading to the suggestion that AI-2 may function as a signalling molecule (Kendall et al. 2007; Jesudhasan et al. 2010; Hirano et al. 2012). *Phototrhobdus*, as is the case with many bacteria, has AI-2 receptors that bind to the secreted molecule and, through a link with AI-2 uptake systems, actively transport AI-2 back into the cell (Krin et al. 2006). In *Phototrhobdus*, AI-2 has been implicated in the production of the carbapenem antibiotic (produced by genes found in the *cpm* operon) where a *luxS* deletion mutant was shown to express higher levels of *cpm* mRNA throughout growth (Derzelle et al. 2002). Transcriptomic analysis also revealed differences in the expression of >100 genes in the *luxS* mutant when compared to wild-type *P. luminescens* TTO1 (Krin et al. 2006). Interestingly, the *luxS* mutant produced less luminescence than the wild-type and this was attributed to the increased production of polyamines in the mutant. Moreover, the *luxS* mutant was more sensitive to oxidative stress, formed reduced biofilms and was hyper-motile when compared to the wild-type (Krin et al. 2006). Interestingly, the BarA-UvrY 2CP in *Phototrhobdus* is required for maximal production of AI-2 and some of the phenotypes reported in the *uvrY* mutant (e.g. reduced bioluminescence) could be rescued by the addition of exogenous AI-2 (Krin et al. 2008). Therefore, AI-2 has pleiotropic roles in *Phototrhobdus* that are linked to secondary metabolism and stress resistance.

5.2 Pathway-specific Regulators

In contrast to work described here on the global regulators of secondary metabolism in *Phototrhobdus*, very little work has been done to identify regulators of specific secondary metabolic pathways, i.e. pathway-specific regulators. This type of

regulatory network topology, whereby master or global regulators work with pathway-specific regulators to ensure that the expression of secondary metabolites is appropriate, is common in bacteria.

5.2.1 AQ

The AQ pigment is produced by proteins encoded by the 9-gene *antA-I* locus (Brachmann et al. 2007). Genes at both ends of this locus (*plu4185* and *plu4195*) are predicted to encode transcriptional regulators although any role for these genes in the regulation of AQ production remains to be established. Another transcriptional regulator, HdfR, was shown to act as a repressor of *antA-I* expression and AQ production (Easom and Clarke 2012). The *hdfR* gene was originally identified during a screen for mutants unable to colonize the IJ nematode and the role, if any, of AQ during nematode colonization is unclear.

5.2.2 Stilbene

The stilbene molecule produced by all *Photorhabdus* strains is a multipotent molecule with confirmed roles in pathogenicity and mutualism. The biosynthetic pathway of this molecule has, for the most part, been elucidated and involves proteins encoded by genes located in at least 4 different genetic loci (Joyce et al. 2008). Therefore, it is possible that the different loci may be regulated independently from each other and flux towards stilbene synthesis may involve both genetic and allosteric regulation. This is more likely when one considers that some of the loci, in addition to having a role in stilbene biosynthesis, are also required for the biosynthesis of fatty acids required during growth, i.e. the *bkdABC* operon encoding a branched-chain amino acid dehydrogenase (Brachmann et al. 2012b). As previously mentioned, the first committed step in stilbene biosynthesis is the conversion of phenylalanine to CA by StlA. Nutrient limitation was recently shown to positively regulate *stlA* gene expression and this was mediated by Lrp, TyrR and σ^S (Lango-Scholey et al. 2013). TyrR was shown to be essential for *stlA* expression (and stilbene production) and, in *Escherichia coli*, TyrR activity is dependent on aromatic amino acids such as phenylalanine (Pittard and Davidson 1991; Lango-Scholey et al. 2013). Therefore, the role of TyrR in *Photorhabdus* may be to couple *stlA* expression to the presence of phenylalanine. In contrast whilst both Lrp and σ^S were required for optimal *stlA* expression in *P. luminescens* TTO1 both of these regulators were dispensable for normal stilbene production (Lango-Scholey et al. 2013). This apparent paradox can be simply explained as *Photorhabdus* produces (and secretes) more CA than is required for stilbene production

(Chalabaev et al. 2008). Therefore, a reduction in CA production due to lower levels of *stlA* expression does not necessarily have to result in a reduction in stilbene production. Interestingly, phenylalanine cannot be used as a carbon source by *Photorhabdus* whilst CA can be assimilated through the activity of the Hca/Mhp pathway (Chalabaev et al. 2008). Therefore, the induction of *stlA* expression following nutrient limitation will, in addition to allowing stilbene production, facilitate optimal nutrient scavenging by *Photorhabdus* in the nutrient limited batch environment of the insect cadaver.

5.2.3 Bioluminescence

Bacterial bioluminescence has been well studied in marine bacteria such as *Vibrio fischeri* and *Vibrio harveyi* where light production has been known for a long time to be regulated by AHL-based quorum-sensing pathways (Meighen 1991). However, *Photorhabdus*, the only known terrestrial bioluminescent bacterium, does not have any AHL-based regulatory circuits. All bioluminescent bacteria have a *lux* operon that contains the 5 genes required for light production, *luxCDABE*. The *luxA* and *luxB* genes encode the light-producing enzyme luciferase and the *luxC* and *luxD* genes encode a fatty acid reductase that produces the aldehyde substrate of luciferase. Light production by *Photorhabdus* increases significantly during the post-exponential phase of bacterial growth suggesting that bioluminescence may have a role during the mutualistic association with the nematode (Schmidt et al. 1989). However, the role and regulation of bioluminescence in *Photorhabdus* is unclear. The expression of the *lux* operon in *P. luminescens* TTO1 is reduced in the *uvrY* mutant. However, the reduction in light production in the *uvrY* mutant can be partially rescued by the exogenous addition of AI-2 to the culture medium and this has been shown to be mediated by the *CsrA/csrB* regulatory system (Krin et al. 2008). Therefore, there may be a significant post-transcriptional component to the regulation of light production in *Photorhabdus*. Interestingly early reports, based on Northern blotting, suggested that the *lux* operon was expressed to the same level in both primary and secondary variants although the level of light production was greatly reduced in the secondary variant (Wang and Dowds 1991). In addition to employing systems based on small regulatory RNA molecules, post-transcriptional regulation can be mediated by the availability of substrate. Acylated urea metabolites (phurealipids) are produced by *Photorhabdus* and have been shown to be antagonists of insect juvenile hormone epoxide hydrolase. Production of phurealipids is dependent on a carbamoyltransferase encoded by *pliA* and disruption of *pliA* completely blocks the production of phurealipids (Nollmann et al. 2015). The substrate for PliA is a fatty acid-derived aldehyde and in independent (and unpublished) experiments mutations in *pliA* were also shown to result in

hyper-bioluminescence (Lea Lango-Schooley and David J. Clarke, unpublished data). Therefore, it is possible that bioluminescence may be controlled, at least in part, by limiting the availability of the fatty acid-derived aldehydes required by the light-producing enzyme, luciferase.

6 Conclusions

P. luminescens TTO1 employs a complex regulatory network to control secondary metabolism during a life cycle that involves significant functional heterogeneity through phenotypic and phase variation and contrasting interactions with different invertebrate hosts. The topology of this network is such that global and pathway-specific regulators interact with primary metabolism to temporally regulate secondary metabolism, including bioluminescence and the production of small bioactive metabolites (see Fig. 4). The secondary metabolites produced during the post-exponential phase of *Photorhabdus* growth have been linked with a role in the mutualistic association with the nematode. Nutrient limitation (through the production of the alarmone (p)ppGpp) has been shown to be a major environmental factor that controls secondary metabolism in *Photorhabdus*. The production of (p)ppGpp is an internal signal that coordinates the transduction of nutrient limitation into a global regulatory response that, directly or indirectly and through the activities of a number of regulators, initiates the transition from pathogenicity to mutualism. Further unravelling of the topology of these regulatory pathways remains an important ambition for a better understanding of the tripartite interaction between *Photorhabdus*, the insect and the nematode.

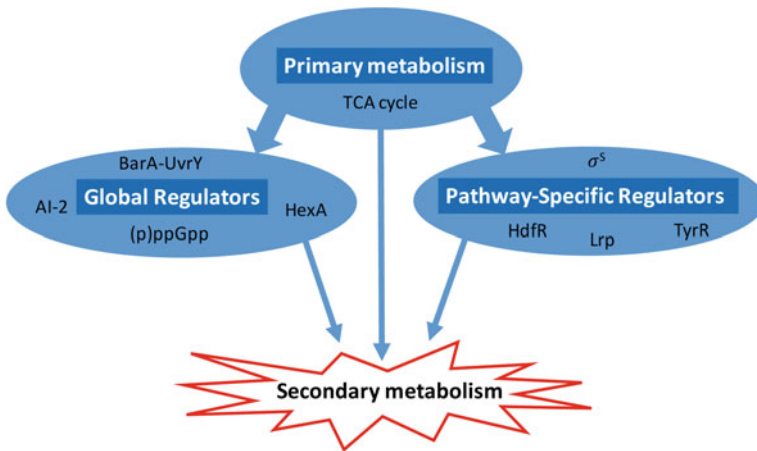


Fig. 4 Regulatory network controlling secondary metabolism in *P. luminescens* TTO1

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