

ACS Chem Biol. Author manuscript; available in PMC 2011 July 16

Published in final edited form as:

ACS Chem Biol. 2010 July 16; 5(7): 659-665. doi:10.1021/cb100117k.

# Exploiting a Global Regulator for Small Molecule Discovery in Photorhabdus luminescens

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### **Abstract**

Bacterially-produced small molecules demonstrate a remarkable range of structural and functional diversity and include some of our most useful biological probes and therapeutic agents. Annotations of bacterial genomes reveal a large gap between the number of known small molecules and the number of biosynthetic genes/loci that could produce such small molecules, a gap that most likely originates from tight regulatory control by the producing organism. This study coupled a global transcriptional regulator, HexA, to secondary metabolite production in *Photorhabdus luminescens*, a member of the Gammaproteobacteria that participates in a complex symbiosis with nematode worms and insect larvae. HexA is a LysR-type transcriptional repressor, and knocking it out to create a *P. luminescens*  $\Delta hexA$  mutant led to dramatic upregulation of biosynthesized small molecules. Use of this mutant expanded a family of stilbene-derived small molecules, which were known to play important roles in the symbiosis, from three members to at least nine members.

*Photorhabdus luminescens*, a Gammaproteobacterium, uses a functionally diverse suite of secondary metabolites to participate in a complex symbiosis with nematode worms (*Heterorhabditis* spp.) and insect larvae. The bacteria persist quietly in the guts of infective juvenile (IJ) nematodes that hunt insect larvae. When a worm succeeds in entering its prey's circulatory system (hemolymph), it regurgitates the bacteria, which then proceed to make toxins that kill the larva, proteases and esterases that liquefy the larva's interior, signals that cause the IJ worms to become reproducing adults, molecules that counter insect defense mechanisms, and molecules that protect their prey from competing bacteria and fungi. Some of the small molecules produced by *P. luminescens* have been identified (1), but despite efforts in many laboratories, these known small molecules represent only a small fraction of the bacteria's metabolic potential. The sequenced *P. luminescens* genome contains at least 33 genes in 20 loci that encode proteins similar to polyketide synthases, nonribosomal peptide synthetases, and  $\beta$ -lactam-producing enzymes (2). The genomic potential for secondary metabolism seen in *P. luminescens* rivals members of the *Streptomyces* genus, the most productive antibiotic-producing bacterial genus (3).

To access these uncharacterized small molecules, we searched for the molecular signals and their targets that control *P. luminescens* metabolism. Recently we reported that the bacteria respond to the high concentrations of L-proline in insect hemolymph by initiating a profound upregulation of secondary metabolite production (4). L-proline enhances the production of small molecules known to be involved in antibiosis, insect virulence, and nematode mutualism along with many structurally and functionally uncharacterized

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molecules. L-proline acts both as an osmoprotectant in the high solute concentrations characteristic of insect hemolymph (5) and more importantly, as a nutrient signal and electron source to enhance the proton motive force believed to regulate downstream pathways involved in antibiotic production and virulence (4). This report concerns the downstream regulation of metabolite production and the discovery of previously undescribed small molecules involved in important aspects of the symbiosis.

Global regulators, which affect the transcription of gene ensembles via regulatory cascades, typically govern the production of small molecules in bacteria (6). Identification and manipulation of these global regulators could provide a powerful approach to complete sets of biologically important and previously uncharacterized small molecules.

### UvrY and Lrp do not regulate stilbene production in P. luminescens

To define a link between the L-proline response and the global regulatory genes involved in controlling the transition from nematode symbiosis to insect pathogenesis, we investigated three candidate genes. The first involved a two-component regulatory system, BarA/UvrY, that regulates a selection of virulence genes. A uvrY-deficient P. luminescens mutant exhibited decreased production of proteases and toxins, as well as decreased bioluminescence (7). UvrY also regulated several genes with suspected roles in antibiotic synthesis and efflux, as well as motility and oxidative stress response (7). However, metabolomic profiling of organic extracts from the uvrY-deficient strain showed no significant changes compared to wild type (WT) in the production of known antibiotics and small molecule virulence factors – anthraquinone polyketides and stilbenes (Figure S1). The second candidate was a leucine-responsive protein (Lrp) regulator that the Goodrich-Blair laboratory had identified as a global regulator of metabolic switching in Xenorhabdus nematophila (8), a bacterium that participates in a symbiosis similar to that of Photorhabdus. When Lrp binds a small molecule ligand, often an amino acid, it becomes a transcriptional activator. Markerless deletion of the homologous lrp gene in P. luminescens by allelic exchange mutagenesis did not result in significant changes to anthraquinone or stilbene production compared to WT (Figure S1). Since earlier work had shown that both Photorhabdus and Xenorhabdus used L-proline to initiate the metabolic switch, this difference in downstream regulation fits a convergent evolution model for the *Photorhabdus* and Xenorhabdus systems (4).

# HexA regulates stilbene virulence factor production in P. luminescens

The third candidate was the LysR-type transcriptional regulator HexA. The Clarke laboratory had shown that the related species *Photorhabdus temperata* uses HexA to repress general antibiotic activity while dwelling within its nematode host (9). Indeed, disruption of this gene, homologous to the *hexA* (hyperproduction of exoenzymes) gene of *Erwinia carotovora*, (10) led to increased (derepressed) antibacterial activity in *P. temperata* as judged by a larger zone of inhibition phenotype (9). Obtaining a stable *hexA* knockout in *P. luminescens* proved challenging in our hands. Insertional inactivation by plasmid integration could be successfully achieved and confirmed by PCR, but the genetic insertion was repeatedly lost in the subculturing attempts needed to obtain a pure mutant strain.

It seemed likely that upregulation of antibiotics and protein toxins in the *hexA* knockout of *P. luminescens* caused the instability, so we reasoned that L-proline might have a protective effect under these conditions, perhaps through the activation of compensatory pathways such as efflux pumps or resistance proteins. By supplementing the medium with 100 mM L-proline, we were able to propagate cultures of the *P. luminescens* insertional *hexA* mutants for chemical interrogation. The stabilizing effect of L-proline in these mutants provides further evidence of its important role in the life cycle of *P. luminescens*.

Metabolomic profiling of organic extracts from the *P. luminescens hexA* mutant by high-pressure liquid chromatography (HPLC) revealed upregulation of multiple metabolites compared to the WT strain (Figure 1). Production of known stilbenes (1–3) in addition to other compounds with stilbene-like UV absorbance was increased in the  $\Delta hexA$  mutant (starred peaks in Figure 1), while production of anthraquinone compounds (10 and 11) was marginally downregulated compared to WT. These differential effects complement those obtained from analysis of the *P. luminescens* proline transporter mutants ( $\Delta proU$  and  $\Delta putP$ ), in which both the  $\Delta proU$  and  $\Delta putP$  strains demonstrated dramatically *increased* production of anthraquinones and *decreased* stilbene production (4). The reciprocal effects between the  $\Delta proU/\Delta putP$  and  $\Delta hexA$  mutants argue that L-proline transport and its subsequent metabolism contributes to derepression of HexA and upregulation of the stilbene class in *P. luminescens*.

A number of the compounds upregulated in the  $\Delta hexA$  strain were isolated and structurally characterized (Figure 2). Analysis of the unknowns by one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HR-MS) led to the discovery of six new stilbene derivatives (4–9). These new compounds mostly share a similar carbon skeleton as the previously identified stilbenes but differ in oxidation states. Because known stilbenes (1–3) mediate several critical functions in the symbiosis, their biosynthesis has been carefully studied (11–13). The identification of six new derivatives in the L-proline stabilized  $\Delta hexA$  mutants illustrates the ability of genetically manipulating global regulators to uncover both the quantitative and qualitative molecular diversity in this important class.

## HexA regulates stilbene and anthraquinone production in P. temperata

To correlate metabolite production to the larger zone of inhibition phenotype previously observed in the P.  $temperata \ \Delta hexA$  mutant (9), we also analyzed organic extracts from the mutant and its WT parent. Differential metabolomic profiling of the mutant compared to WT revealed greatly enhanced production of both stilbenes and anthraquinones in the  $\Delta hexA$  mutant, which was independent of proline concentration (Figure S2). Production of the stilbene metabolites by  $\Delta hexA$  far exceeded that of the WT strain even in cultures supplemented with high L-proline concentrations (Figure S2). Regulation of the major metabolites in the P. temperata mutant resulted in a dramatic increase in production of a small number of compounds, including known stilbenes and anthraquinones. The P. temperata temperata

# L-proline dose-response effects in P. $luminescens \Delta hexA$

To further investigate the effect of increasing L-proline concentration on the production of metabolites by *P. luminescens*  $\Delta hexA$ , L-proline dose-response curves were generated for the upregulated stilbenes (Figure 3). Similar to the effects seen in *P. temperata*  $\Delta hexA$  (Figure S2), *P. luminescens*  $\Delta hexA$  exhibits constitutive production of many of these metabolites compared to WT. Production of the reduced stilbene derivative (4), for example, was upregulated 50-fold in  $\Delta hexA$  with high concentrations of supplemental L-proline. Under high L-proline concentrations, metabolites  $\mathbf{5} - \mathbf{8}$  are repressed in both WT and  $\Delta hexA$ , perhaps due to their conversion to other compounds, suggesting a metabolic shift rather than a general upregulation. Production of  $\mathbf{9}$  is unchanged in the  $\Delta hexA$  mutant (Figure S3), but

this metabolite diverges structurally from the other stilbenes and may serve other biological functions.

## Stilbenes possess multipotent activities

Stilbenes are common plant metabolites, but *Photorhabdus* is the only known producer of stilbenes outside the plant kingdom (14). The stilbenes in *Photorhabdus* are biosynthesized from the condensation of two  $\beta$ -ketoacyl intermediates (Scheme 1), while plant-derived stilbenes arise through the linear elongation of an activated phenylpropanoid starter unit with malonyl-CoA extender units (11), which indicates that the *Photorhabdus* biosynthetic pathway evolved independently of plants. In spite of its independent bacterial origin, the pathway's genes have an odd organization. While bacterial biosynthetic pathways for secondary metabolites, including those for many of the annotated pathways in the *P. luminescens* genome (15), typically have clustered genes, the stilbene biosynthetic genes are not clustered (11). They are organized more like the scattered genes found in plant biosynthetic pathways.

The newly-identified reduced stilbene (4) is especially interesting from both biosynthetic and physiological perspectives. The biosynthesis of 1 proceeds from phenylalanine, derived from prephenate, and leucine (11). Similar transformations on 2,5-dihydrophenylalanine (DHPA) would lead to the synthesis of 4 rather than 1 (Scheme 1). DHPA, also derived from prephenate (16), is a known antibiotic produced by *Streptomyces sp.*, and functions as a microtubule-disrupting agent (17). Compound 4 and any of its proposed precursors could serve as oxidative sinks to protect the bacterium from the massive levels of oxidative stress it encounters in the insect hemolymph (18). Oxidation would yield the corresponding metabolite along the pathway to 1. DHPA, for example, can spontaneously oxidize to phenylalanine (19). This strategy of producing easily oxidized metabolites resembles the production of redox active pigments by *Pseudomonas aeruginosa* and *Staphylococcus aureus* (20–21) and suggests still another biological role for this stilbene family in addition to those described below (11). The biosyntheses of the remaining new metabolites remain unknown, but they are likely to be oxidation products of the major stilbenes 1 or 2.

The previously described *Photorhabdus* stilbenes (1–3) exhibit a broad range of biological activities that illustrate their critical roles in many aspects of the complex symbiosis. They mediate interactions with competing microbes, with their nematode hosts, and with their insect prey. These stilbenes have been shown to possess antibiotic activity against fungi and Gram-positive bacteria (22), they are potent inhibitors of phenoloxidase, one of the insect's key defenses against microbial pathogens (13), and they are essential in maintaining the bacterium's mutualistic symbiosis with its nematode host by influencing nematode development (11).

In order to efficiently exit their IJ stage and become reproducing adults, the nematodes respond to molecular signals made by their bacterial symbionts (23). Previous investigations have demonstrated that IJ nematodes grown on a stilbene-deficient strain of *P. luminescens*,  $\Delta stlA$  mutants with the first committed step of stilbene biosynthesis knocked out (Scheme 1), have a recovery rate only 5–15% of that achieved with WT *P. luminescens* (11). Growing IJ nematodes on the  $\Delta stlA$  strain supplemented with either stilbene 1 or the StlA product cinnamic acid yielded almost full IJ recovery, while no recovery was observed when IJs were supplemented with 1 or cinnamic acid but no bacteria (11). These results indicate that stilbene-derived compounds are essential for nematode recovery, but stilbene 1 cannot induce recovery on its own. It is likely that 1 is converted by *P. luminescens* (and possibly further elaborated by the nematode) to the biologically active but not yet identified compound.

The IJ stage of the nematode *H. bacteriophora* is analogous to the dauer stage of the much better known model nematode *Caenorhabditis elegans*. In *C. elegans*, entry into dauer is mediated by the dauer pheromone, which is made by the nematodes. Exit from dauer is governed by an unknown molecular signal called the 'food signal' (24) because it is made by the bacteria that *C. elegans* consume. While dauer pheromones for *C. elegans* have been actively studied, the food signal has been ignored, and access to the new stilbenes could provide the molecular tools needed to study this developmental switch. Further biological experiments will be needed to fully explore the roles played by individual members of the stilbene family uncovered in this study.

#### Conclusion

Identifying the small molecules produced by orphan biosynthetic pathways – pathways that can be identified in sequenced genomes but whose products have not been characterized – represents both a great opportunity and a substantial challenge. The cryptic metabolites produced by these pathways typically outnumber the known metabolites by an order of magnitude, and many would be expected to have potential therapeutic applications. These metabolites are most likely cryptic because the pathways that produce them are tightly regulated, and we currently know very little about what conditions activate them. This study shows that identifying global regulators and manipulating them can lead to the accelerated discovery of cryptic metabolites. The study also illustrates the power of focusing on symbiotic associations both as a way to identify regulatory triggers and as a way to place any metabolites that might be produced into their biological context.

#### **Methods**

### Genetic inactivation of Irp and hexA in P. luminescens

*P. luminescens* gDNA was isolated as previously described (25). The entire coding sequence from start to stop codons of lrp (locus tag: Plu1600; Protein Accession: NP\_928891) was excised by allelic-exchange mutagenesis to generate a markerless deletion mutant. The exchange sequence for lrp consisted of ~1 kB of upstream and downstream genome sequence fused by overlap extension PCR (26) (see Supplemental Methods). The full-length lrp exchange sequence was digested with SacI, inserted into the corresponding site in pDS132 (27), and verified by restriction analysis (pDiLrp). Cloning was carried out in E. coli strain WM3618 lambda pir.

Because markerless deletion attempts of *hexA* failed in our hands, an internal *hexA* (locus tag: Plu3090; Protein Accession NP\_930322) gene fragment was amplified, digested with SacI, and inserted into the corresponding site in pDS132 (pDiHexA) for plasmid integration (see Supplemental Methods). Ligation products in both directions were successfully taken forward to insertionally inactivate *hexA* by pDiHexA plasmid integration containing a chloramphenicol resistance marker.

Mutants were generated using similar procedures as previously described (4). The pDS132 deletion constructs (pDiLrp or pDiHexA) were transformed into the diaminopimelic acid (dap) auxotroph donor strain, *E. coli* WM6026 lambda pir (28), by heat-shock transformation (29). The donor *E. coli* and recipient WT *P. luminescens* TT01 were filter mated, replated on LB-chloramphenicol, then selected on LB sucrose plates for counterselection (see Supplemental Methods). Positive deletions were identified by colony PCR and sequence verified. For insertional inactivation of *hexA*, agar plates used for filter mating and all subsequent plating steps were also supplemented with 100 mM L-proline. Successful *hexA* plasmid integrants were identified by colony PCR and sequence verified (see Supplemental Methods). No SacB counter selection was performed.

#### P. temperata \( \Delta hexA \) proline dose-response

Rifampicin-resistant  $\Delta hexA$  and its rifampicin-resistant parent strain of *P. temperata* were assessed for metabolite stimulation with increasing concentrations of L-proline (0–100 mM) (9). All experimental conditions were identical to those previously described (4).

#### P. luminescens \( \Delta hexA \) proline dose-response

The *P. luminescens*  $\Delta hexA$  mutant was grown on LB agar + 100 mM L-proline + 25  $\mu g$  mL<sup>-1</sup> chloramphenicol for two days at 30 °C. The WT strain was cultured similarly, but without antibiotic. Single colonies were selected and grown two additional days in 5 mL LB broth (WT) or LB with 25  $\mu g$  mL<sup>-1</sup> chloramphenicol ( $\Delta hexA$ ). Cultures were then centrifuged and resuspended in 5 mL fresh LB (to remove chloramphenicol and ensure medium consistency between mutant and WT). For metabolite stimulation assays, 50  $\mu$ L of this resuspended culture was used to inoculate 5 mL of a rich tryptone-yeast extract based medium (2 g tryptone, 5 g yeast extract, and 10 g NaCl per L) with increasing amounts of L-proline (0–100 mM). Triplicate cultures were grown to stationary phase over 72 h at 30 °C and 250 rpm. The cultures were vigorously extracted with 6 mL ethyl acetate, then centrifuged and 4 mL of the top organic layer dried. These dried extracts were resuspended in 1 mL methanol, and 50  $\mu$ L of this mixture was injected for HPLC analysis to quantify metabolite production (see Supplemental Methods for details).

Due to the tendency of the *hexA* mutant to revert to WT in the absence of proline, we reasoned that cultures supplemented with little to no proline may similarly have reverted during the 3-day incubation prior to extraction for metabolite analysis, thereby skewing the results. Prior to extraction for assessment of metabolite production, representative cultures with high and low concentrations of proline were therefore sampled and used as template in a PCR assay to evaluate whether reversion to WT was occurring. Results demonstrated only low levels of reversion in cultures with no supplementary proline over the 3-day growth period (Figure S4).

### NMR analysis

NMR experiments (Varian: <sup>1</sup>H, gCOSY, gHSQC, and gHMBC) were performed in deuterated methanol with a symmetrical NMR microtube susceptibility-matched with the solvent (Shigemi, Inc.) on a Varian INOVA 600 MHz NMR. Known metabolites were confirmed by <sup>1</sup>H NMR and mass spectrometry.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

## **Acknowledgments**

This work was supported by National Institutes of Health grant R01 GM086258 to J.C. J.M.C. is a Damon Runyon Fellow supported by the Damon Runyon Cancer Research Foundation (DRG-2002–09). We thank D.J. Clarke (University College Cork, Ireland) and A. Danchin and E. Krin (Institut Pasteur, France) for the *P. temperata*  $\Delta hexA$  and parent strains and the *P. luminescens* (Pl2105)  $\Delta uvrY$  strain, respectively. We also thank the University of Illinois MS service lab for HR-MS measurements.

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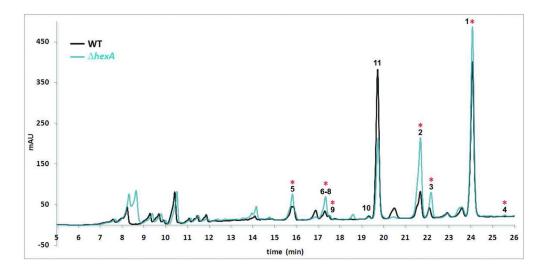


Figure 1. Representative HPLC trace overlay (210 nm) of organic extracts of WT (black) and  $\Delta hexA$  (blue) *P. luminescens* cultures, both grown in 5 mM L-proline. Starred peaks denote stilbene derivatives, upregulated in the  $\Delta hexA$  strain. Numbers above peaks refer to compounds in Figure 2.

Figure 2. Structures of new and known stilbene derivatives isolated from P. luminescens. 1-3 have been previously isolated from Photorhabdus. 4-9 are new natural products. All four stereoisomers of 5 were isolated in a single fraction. 9 was previously described as a side product in a synthetic scheme (30).

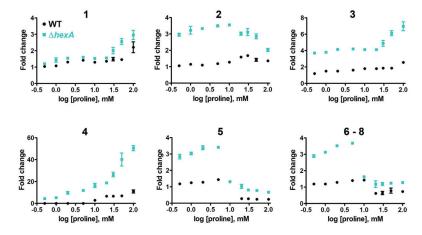


Figure 3. Fold change in production of stilbene metabolites by WT (black •) and  $\triangle hexA$  (blue •) P. *luminescens* with increasing concentrations of supplementary L-proline. Numbers above curves refer to structures in Figure 2. Compounds 6 - 8 are overlapped on the HPLC trace and were therefore integrated together.

#### Scheme 1.

Proposed biosynthesis of reduced stilbene 4, based on the established biosynthesis of 1. The pathways to these compounds diverge at prephenate, and oxidation of any intermediate along the route to 4 leads to the convergent production of 1.