The Global Regulators Lrp, LeuO, and HexA Control Secondary Metabolism in Entomopathogenic Bacteria

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Photorhabdus luminescens TTO1 and Xenorhabdus nematophila HGB081 are insect pathogenic bacteria and producers of various structurally diverse bioactive natural products. In these entomopathogenic bacteria we investigated the role of the global regulators Lrp, LeuO, and HexA in the production of natural products. Lrp is a general activator of natural product biosynthesis in X. nematophila and for most compounds in TTO1. Microarray analysis confirmed these results in X. nematophila and enabled the identification of additional biosynthesis gene clusters (BGC) regulated by Lrp. Moreover, when promoters of two X. nematophila BGC were analyzed, transcriptional activation by Lrp was observed. In contrast, LeuO in X. nematophila and P. luminescens has both repressing and activating features, depending on the natural product examined. Furthermore, heterologous overexpression of leuO from X. nematophila in the closely related Xenorhabdus sientirmaii resulted in overproduction of several natural products including novel compounds. The presented findings could be of importance for establishing a tool for overproduction of secondary metabolites and subsequent identification of novel compounds.

Keywords: transcription factors, Photorhabdus, Xenorhabdus, natural product, regulation of natural products

INTRODUCTION

Bacterial natural products are of great importance for our current health system and the development of new therapeutic agents or plant protectants (Zhou et al., 2008; Newman and Cragg, 2012). The entomopathogenic bacterial genera Photorhabdus and Xenorhabdus are potent producers of structurally diverse compounds (Figure 1) that are important during their mutualistic lifestyle in symbiosis with nematodes, the infection of insect larvae and protection of the host cadaver against competitors (Thomas and Poinar, 1979). Within the last few years, advances in understanding regulation in entomopathogenic bacteria have been made, but a general view regarding natural product biosynthesis and function is missing.

Lrp (leucine responsive protein) and LeuO have both been described as global regulators of transcription in Escherichia coli (Tani et al., 2002; Shimada et al., 2009) and Salmonella enterica (Baek et al., 2009; Dillon et al., 2012). Lrp type regulators are widespread in the bacterial world.
FIGURE 1 | Examples of natural products from *P. luminescens* TTO1, *X. nematophila* HGB081, and *X. szentirmaii*.
LeuO belongs to the largest family of transcriptional regulators in prokaryotes, the LysR type transcriptional regulators (Pareja et al., 2006; Hernández-Lucas and Calva, 2012). In *S. enterica*, where it was first described (Hertzig et al., 1980; Henikoff et al., 1988), it indirectly acts as a repressor for SPI-1 (Espinosa and Casadesús, 2014) and is often described as an antagonist of heat-stable nucleoid-structure protein (H-N5) (Chen and Wu, 2005). In *Vibrio cholerae*, however, LeuO is part of the ToxR regulon and down-regulates important virulence factors. The expression of *leuO* in *V. cholerae* is activated by the natural product cyclo(Phe-Pro) (Bina et al., 2013). In *Vibrio parahaemolyticus* a LeuO homolog is positively controlled by ToxRS and negatively regulates transcription of genes in the pathogenicity island 1 (SPI-1) and negatively regulates transcription of *lrp* (Whitaker et al., 2012). As the production of secondary metabolites in *X. nematophila* and *Photorhabdus luminescens* is important for virulence and LeuO is described as a regulator for virulence factors in other bacterial species, we investigated whether LeuO also plays a role in the regulation of natural products in these entomopathogenic bacteria.

HexA is a transcriptional repressor of the LysR type family. In *Photorhabdus temperata* it has an important role in the interaction with the nematode by repressing antibiotic production within the host (Joyce and Clarke, 2003). Kontnik et al. (2010) showed that ΔhexA mutants of *P. temperata* and *P. luminescens* produce significantly more IPS and derivatives thereof compared to the wild type while anthraquinone production is upregulated in the *P. temperata* mutant but downregulated in *P. luminescens* ΔhexA. Recently it was observed that hexA is part of a regulatory cascade in which it is controlled by Hfq. Deletion of hexA restores secondary metabolite production in a Δhqf mutant which otherwise shows very little production (Tobias et al., 2016).

In this study, the influence of the three global regulators LeuO, Lrp, and HexA with the focus on secondary metabolite production is described in *P. luminescens* and *X. nematophila*. Furthermore, we examine the potential for heterologous expression of LeuO to modulate natural product production in *Xenorhabdus szentirmaii*.

**MATERIALS AND METHODS**

**Bacterial Strains**

The strains used in this work are *E. coli* S17-1 λpir (Tpγ Smγ recA thi hsdR RP4-2-Tc::MuKm::Tn7, λpir phage lysogen), *E. coli* DH10B (Life Technologies), *P. luminescens* TTO1 (rifampicin resistant strain) (Duchaud et al., 2003), *X. nematophila* ATCC 19061 (HGB800), *X. nematophila* AN6/1 (HGB801, rifampicin resistant strain, isolated by S. Forst), *X. nematophila lrp-2*Km (HGB1059) (Cowles et al., 2007), and *X. szentirmaii* DSM 16338 (Lengyel et al., 2005).

**Cultivation of Bacteria**

Cells were generally grown in lysogeny broth (LB) with 0.5% (w/v) NaCl or in SF-900 broth (Gibco™) at 30°C or 37°C (for *E. coli*), 200 rpm in Erlenmeyer flasks for liquid cultures. LB plates contained 1.5% agar. Cultures for extraction were inoculated from overnight pre-cultures with a starting OD600 of 0.1. For induction of *pBAD*, cultures were supplemented with 0.2% (w/v) L-arabinose. Concentrations for antibiotics, added when necessary, were kanamycin (50 µg/mL), rifampicin (50 µg/mL), and chloramphenicol (20 µg/mL). PCR and Sanger DNA sequencing were used to verify mutants.

**Construction of Plasmids**

Phusion polymerase (Finnzymes/Thermo Scientific) was used for amplification of PCR products and plasmids were extracted using a Miniprep Kit (Thermo Scientific). Plasmids and purified PCR-products were digested with the described restriction enzymes (Fermentas/Thermo Scientific), purified by gel electrophoresis, and ligated and transformed into *E. coli* via electroporation.

The pBAD30 plasmid (Guzman et al., 1995) was amplified without *bla* via PCR (oligonucleotides: forward, 5′-CCATGGCATA TATACTTTAG ATTGATTTACG-3′, reverse, 5′-CTCGAGGATA ATAATGGTTT CTTAGACG-3′) and blunt-end ligated with *P_anmp-kanR* (oligonucleotides: forward, 5′-CCTGAGGATA ATAATGGTTT CTTAGACG-3′; reverse, 5′-CCATGGACT TGGTCTGACA GTTACC-3′) resulting in pBAD30_ kanR (p15A, araC, ara02, and ara01 sites, *P_BAD*, *kanR*; details and DNA sequence are depicted in the Supplementary Table S1). Regulator genes *leuO* (TTO1: pled3672, 945 bp; HGB801: XNC1_1548, 948 bp) and *lrp* (TTO1: plu1600, 495 bp; HGB801: XNC1_1548, 495 bp) were amplified with overhangs for restriction enzyme sites KpnI and Pael from genomic DNA (extracted with Qiagen Puregene Yeast/Bact. Kit) and subcloned in pJET1.2 (Life Technologies). These restriction enzyme sites were used for ligating the fragments into the corresponding sites of pBAD30_ kanR. *E. coli* DH10B was transformed with this plasmid and LB kanamycin plates were used to screen for positive clones.

For the construction of cluster expression plasmid (pCEP) plasmids, the start of the genes *leuO*, *lrp*, and *hexA* from *P. luminescens* TTO1 (oligonucleotides for *leuO*: forward, 5′-CAT ATGGGTT AGATAACACTC AGTA AACTG-3′; reverse, 5′-CTC GAGGGA ATGATGAAAAT CCA-3′, 547 bp;
oligonucleotides for lrp: forward, 5′-CATATGATAG ATAATAAA AAA ACCTCGGGGA AAAGATC-3′; reverse, 5′-CTCGAGCTG GG CAAACCGCAAC AAAG-3′, 427 bp; oligonucleotides for hexA: forward, 5′-CATATGTTAAA TGCAATCTG CGCAT AATG-3′; reverse, 5′-CTCGAGTGGC ATAATACCG GTTTG-3′, 519 bp) and X. nematophila HGB081 (oligonucleotides for leuO: forward, 5′-CATATGACTG GATAACACTG GTGATCC-3′; reverse, 5′-CTCGAGGTTA ATACGTGTTG ACACACTCA TTTCC-3′, 517 bp; oligonucleotides for lrp: forward, 5′-CATATGATTTG ATATAAGAA GCGTCCAGGA AAAG-3′; reverse, 5′-CTCGAGCTCT GTTTTACTTC TTACAACAA CATAAGTGC-3′, 476 bp) were amplified from genomic DNA with overhangs for NdeI and Xhol (bold) and subcloned into pET12 (Life Technologies). After digestion with the corresponding enzymes and clean up (gel electrophoresis and extraction), the fragments were cloned into the corresponding sites of the previously described pCEP vector (Bode et al., 2015). Electrocompetent S17-1 λpir cells were transformed with these plasmids. Positive clones (selected on chloramphenicol plates) were then used for conjugation, so that the plasmid could be integrated into P. luminescens TTO1 and X. nematophila HGB081 by homologous recombination. Selective media containing rifampicin and chloramphenicol were used for identification of positive clones. The resulting four mutant strains, P. luminescens P
\[\text{RAD}-\text{leuO}\]
\[\text{or } P\text{RAD}-\text{lbp} \text{ and X. nematophila P}\]
\[\text{RAD}-\text{leuO} \text{ or } P\text{RAD}-\text{lbp} \text{ were verified by PCR and sequencing.}

To create promoter activity reporters for the gene clusters encoding xenematide (xene) or xenortide (xenor) biosynthesis enzymes, vector pYEY2.2 (details and DNA sequence are depicted in the Supplementary Table S2) was constructed by exchanging the reporter gene gfpmut3.1 on pFU69 (Uliczka et al., 2011) with the reporter gene ypet, encoding an optimized yellow fluorescent protein (Nguyen and Daugherty, 2005) (Life Technologies). ypet as well as the kanR resistance cassette were cloned into low copy vector pFU99 (Uliczka et al., 2011), thereby replacing the lacZ and cmR genes and generating pYEY2.2 (pSCL01*, kanR, ypet). Promoter regions P
\[\text{Xene} \text{ (oligonucleotides: forward, 5′-ATATCCTCGAG CGGCTTACAT CATCAACGCCA-3′ and reverse, 5′-ATATGCTAGC AAATATTTACGGAAGCAA-3′, 446 bp) and P}\]
\[\text{Xenor} \text{ (oligonucleotides: forward, 5′-ATATCCTCGAG CTCAACATTATAACACGCTCTCTCTCC-3′ and reverse, 5′-ATATGCTAGC ATGTTTCTCTT TACTACCTTCT GTG-3′, 392 bp) were amplified with overhangs for Xhol and Nhel (bold). The corresponding restriction enzyme sites were then used for insertion of the promoter regions into the pYEY2.2 vector. E. coli DH10B was used for these cloning procedures.}

Transformation of Xenorhabdus Strains
Promoter reporter and overexpression plasmids were transformed into X. nematophila and X. szentirmaii via heat shock transformation as described previously (Xu et al., 1989). For this purpose, cultures were grown to OD
\[\text{600} \sim 0.6 \text{ and 1 mL culture was harvested for each individual transformation. Positive clones were selected on selective media according to antibiotic resistance encoded on the vector.}

### Extraction of Natural Products from Bacterial Cultures
For direct extraction of cultures, cells were inoculated with OD
\[\text{600} \sim 0.1 \text{ in SF-900 broth (Gibco™) (or sorted and inoculated in LB for the FACS analysis) and incubated for 3 days at 30°C, 200 rpm. Subsequently, equal amounts of culture and methanol were mixed. After centrifugation the supernatant was directly analyzed via high performance liquid chromatography coupled with mass spectrometry (HPLC-MS).}

Cultures for the heterologous leuO overexpression in X. szentirmaii were inoculated with OD
\[\text{600} \sim 0.1 \text{ in LB, supplemented with 2% (v/v) Amberlite XAD-16 and harvested after 3 days of cultivation by decanting cells and supernatant. The XAD-16 beads were extracted with methanol with one culture volume for 1 h while stirring. After filtration the extract was evaporated to dryness. For HPLC-MS analysis the extract was redissolved in MeOH in the original culture volume.}

### HPLC-MS Analysis
Extracts were analyzed using the UltiMate 3000 LC System ( Dionex) coupled to an amaZon X ion trap with electrospray ionization (Bruker Daltonics). A water/acetonitrile gradient with 0.1% formic acid as mobile phase with 0.4 mL/min flow separated the compounds on a C18 column (ACQUITY UPLC BEH, 1.7 mm, Waters). Relative amounts were quantified either directly by integration of the area peak in DataAnalysis 4.2 Software (Bruker Daltonics) or by using Target Analysis (Bruker Daltonics) with the recently described setup (Ahrendt et al., 2015). The m/z ratios that were used for this procedure are as follows: m/z 215.2 [M+H]+ (desmethylphurealipid A), m/z 586.4 [M+H]+ (GameXPeptide A), m/z 552.4 [M+H]+ (GameXPeptide C) [M+H]+, m/z 255.1 [M+H]+ (IPS), m/z 334.7 [M+2H]+2+ (mevalamcapeptide), m/z 273.2 [M+H]+ (nematophin), m/z 229.2 [M+H]+ (phurealipid D), m/z 295.2 [M+H]+ (photorynone D), m/z 574.4 [M+H]+ (rhabdopeptide 1), m/z 838.4 [M+H]+ (szentiamide), m/z 663.3 [M+H]+ (xenematide A), m/z 466.3 [M+H]+ (xenocoumacin 1), m/z 281.1 [M+H]+ (xenofuranone A), and m/z 410.3 [M+H]+ (xenortide A). All analyses were performed in triplicate. Base peak chromatograms and extracted ion chromatograms (EICs) displayed in some figures were also created with DataAnalysis 4.2 (Bruker Daltonics).

### FACS Analysis
Fluorescence activated cell sorting (FACS) analysis and cell sorting were performed on a FACS AriaIII cell sorter (BD Biosciences). YPet was excited with a 488 nm Laser. The filter peak chromatograms and extracted ion chromatograms (EICs) displayed in some figures were also created with DataAnalysis 4.2 (Bruker Daltonics).
recorded with the same instrumental adjustments. At the same time samples for direct HPLC-MS analysis were collected.

**Microarray Analysis**

Tiled microarrays were used to measure transcript levels in *X. nematophila* wild type (HGB800) and *lrp-2::Km* early stationary phase cells as previously reported (Hussa et al., 2015) to compare the expression levels of biosynthesis genes. Briefly, the average signal strength of all probes was used as the baseline signal strength level and genes with average signal strength (across probes within that gene) of at least five times this amount were considered expressed. For these genes, the baseline signal strength value was subtracted from the average signal strength for the gene, and all genes were normalized across strains using the values for the *recA* gene. Differences between strains in normalized values of at least twofold were considered differentially expressed. Data shown in Table 1 represent a subset of genes from this analysis.

**RESULTS**

**Effects of Lrp, LeuO, and HexA on Selected Natural Products in Photorhabdus and Xenorhabdus**

To investigate the effects of the global regulators Lrp, LeuO, and HexA on secondary metabolism we created mutants in which the natural promoter of each of these global regulators was exchanged with the inducible P_BAD promoter via homologous recombination using the recently described, integrative pCER vector (Bode et al., 2015). The P_BAD promoter is tightly regulated in *Xenorhabdus* and *Photorhabdus* (Bode et al., 2015), allowing us to control the levels of LeuO or Lrp expressed (either no expression or induced expression) and study downstream regulatory effects. The resulting mutants were grown for 3 days in SF-900 broth, which is a medium for cultivation of insect cell lines and should therefore provide growth conditions that are closer to natural hosts than LB. Extracts were obtained using methanol directly from the cultures and analyzed by HPLC-MS as described in Section “Materials and Methods.” The resulting production was divided by the OD$_{600}$ of the cultures as we observed that some induced and non-induced cultures showed slight differences in their OD$_{600}$ relative to the non-modified wild type cultures (induced: *P. luminescens* P_BAD-lrp: 0.8×, P_BAD-leuO: 1.1×, P_BAD-hexA: 0.7×, *X. nematophila* P_BAD-lrp: 1×, P_BAD-leuO: 0.7×; non-induced: *P. luminescens* P_BAD-lrp: 1.2×, P_BAD-leuO: 0.8×, P_BAD-hexA: 0.6×, *X. nematophila* P_BAD-lrp: 0.8×, P_BAD-leuO: 1.1×). We found that *P. luminescens* Lrp and LeuO both had either positive or a negative effects on metabolite production, depending on the natural product class while HexA decreased production for all of these compounds (Figure 2A).

In the non-induced *P. luminescens* P_BAD-lrp and P_BAD-hexA strains, we detected four and five times more mevalagmapeptide (Bode et al., 2015) compared to either the wild type or the induced P_BAD-lrp and P_BAD-hexA strains, respectively. Thus, Lrp and HexA act as negative regulators of mevalagmapeptide biosynthesis. In contrast, *leuO* induction had no influence on mevalagmapeptide production. Wild type cultures had higher levels of several compounds including photopyrone D (Nollmann et al., 2015b) and GameXPeptide A (Bode et al., 2012) compared to the non-induced P_BAD-lrp culture, suggesting a role as an activator. However, the induced cultures failed to reach wild type levels (GameXPeptide). The amount of phurealipid A in the induced P_BAD-lrp strain is also lower than in the wild type, however, its precursor desmethylphurealipid A (Nollmann et al., 2015b) is produced by the P_BAD-lrp mutant in higher amounts relative to wild type if *lrp* expression is induced. In summary, the data shows that in *P. luminescens*, Lrp positively regulates production of GameXPeptide A and desmethylphurealipid A, activate photopyrone D and phurealipid A production to a lesser extent, and negatively regulates mevalagmapeptide, but does not significantly affect IPS production.

Based on metabolites produced in the induced versus non-induced cultures of the *P. luminescens* P_BAD-leuO mutant, LeuO positively influences GameXPeptide A, and IPS production, while it negatively impacts phurealipid A biosynthesis. Desmethylphurealipid A production is hardly influenced by LeuO and the regulation on photopyrone D is not clearly distinguishable. Therefore, *P. luminescens* LeuO acts as both an activator and repressor.

The induced P_BAD-hexA strain shows strongly decreased production of all compounds that were analyzed while the non-induced strain produces higher amounts of mevalagmapeptide (~5.6×), IPS (~5.7×), and desmethylphurealipid A (~4.7×) compared to the wild type while the amounts of photopyrone D and phurealipid A are lower.

In contrast to *P. luminescens* the effects of *lrp* on secondary metabolites in *X. nematophila* are more dramatic: compared to wild type *X. nematophila*, the non-induced P_BAD-lrp mutant strain produces significantly lower levels (~0.1× to ~0.28×) of the examined secondary metabolites except for

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**TABLE 1 | Summary of regulatory effects of Lrp and LeuO on secondary metabolite production in *P. luminescens* and *X. nematophila***

| Compound | Lrp | LeuO | HexA |
|----------|-----|------|------|
| **Photorhabdus luminescens** | | | |
| Mevalagmapeptide | ↓ | – | ↓ |
| Photopyrone D | ↑ | – | ↓ |
| GameXPeptide A | ↑ | ↑ | ↓ |
| Isopropylstilbene | – | ↑ | ↓ |
| Phurealipid A | ↑ | ↓ | ↓ |
| Desmethylphurealipid A | ↑ | ↓ | ↓ |
| **Xenorhabdus nematophila** | | | |
| Nematin | ↓ | ↓ | |
| Xenematide A | ↑ | ↑ | |
| Xenocoumacin 1 | ↑ | ↓ | |
| Xenoridine A | ↑ | ↓ | – |
| Phabodepeptide 1 | ↑ | ↓ | |
| Xenotetrapiptide | ↓ | ↑ | |

Downward and upward arrows indicate negative and positive regulation by the regulator, respectively, while hyphen marks no significant change. Arrow and hyphen together show a trend, meaning that a clear difference was observable between wild type and non-induced culture but not between induced and non-induced mutant.
**Microarray Analysis**

A previously reported microarray analysis (Hussa et al., 2015) was mined to examine the influence of Lrp on expression levels of genes encoding (or predicted to encode) secondary metabolism regulators in *P. luminescens* and *X. nematophila*. The analyses described above provided an opportunity to search for previously not described natural products that might be produced by the various strains we tested. Toward this end we compared the base peak chromatograms of the wild type and the induced and non-induced *P. luminescens* and *X. nematophila* mutants. In several cases, clear differences in peak intensities were observed among strains. We examined the mass spectra at the same retention times to find the m/z ratios of the compounds that are responsible for the observed differences. Corresponding EICs for those m/z values that do not belong to the already mentioned compounds or their derivatives were generated to display these findings.

**FIGURE 2** Relative production of selected natural products in *P. luminescens* P**BAD-lrp** (lrp), P**BAD-leuO** (leuO), and P**BAD-hexA** (hexA) (A), and *X. nematophila* HGB081 P**BAD-lrp** (lrp) and P**BAD-leuO** (leuO) (B) compared to the respective wild type strains. For induction of P**BAD**, cultures were supplemented with 0.2% arabinose (+). Average production of the triplicates (three individual cultures inoculated from the same preculture) was divided by average OD**600** values and normalized to the appropriate wild type strains without addition of arabinose (−). Error bars indicate the standard deviation. Values for compounds produced in very low amounts are presented above the corresponding bars.
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metabolite biosynthetic activities. Expression levels of genes within 12 different gene clusters were compared between a X. nematophila lrp-2-kan mutant (Cowles et al., 2007) and its wild type parent (Table 2). Consistent with our metabolite analyses described above, these data indicate that Lrp positively influences the expression of genes involved in xenocoumacin, xenotetrapeptide, rhabdopeptides, xenortides, and xenematisides. The genes that encode for PAX-peptide biosynthesis, a compound class that could not be quantified with our HPLC-MS system, were also positively regulated by Lrp. Six additional putative biosynthesis genes or gene clusters of which the biosynthesis product is unknown, also displayed higher levels of expression in wild type relative to the lrp mutant. Only one putative biosynthesis gene (XNC1_2799) displayed negative regulation by Lrp. Overall, the microarray results provide evidence that Lrp influences secondary metabolite production at the level of gene expression (transcription or RNA stability).

Lrp Influences Promoter Activity

To further explore the influence of Lrp on transcription of secondary metabolite gene clusters, we created reporter constructs that placed the yellow-fluorescent-protein YPet under control of the promoters driving expression of Lrp-dependent xenematide A (xene) and xenortide A (xenor) biosynthesis genes: P_{Xene}-ypet and P_{Xenor}-ypet reporters, respectively. These reporters and a negative control (nc, promoterless reporter plasmid) were expressed in X. nematophila pBAD-lrp from low-copy plasmids (pYEH2.2) and analyzed by FACS-analysis under non-inducing and inducing growth conditions. Increased promoter activity, based on YPet fluorescence, was observed on single cell level when lrp was induced with arabinose for P_{xene} and P_{xenor}, suggesting an effect on transcriptional regulation of the corresponding biosynthesis genes by Lrp (Figure 4A). Extracts of the same cultures supports these findings as the induced cultures contain a higher level of the corresponding compounds (Figure 4B).

Effects of Heterologous Expression of leuO on Metabolite Production

Based on our finding that induced expression of lrp and leuO could reveal previously unidentified compounds, we considered the possibility that their ectopic expression in a heterologous host might allow expanded natural product discovery. To test if LeuO could function to control secondary metabolite production in a heterologous host, we generated plasmids pBAD30_kanR-based arabinose-inducible overexpression plasmids pBAD30_kanR_leuO_P encoding LeuO from P. luminescens and pBAD30_kanR_leuO_X encoding LeuO from X. nematophila. When these were expressed in X. szentirmaii we found that induced expression of X. nematophila leuO had major effects on secondary metabolite production while P. luminescens leuO induction did not visibly alter production of tested compounds (Figure 5). As in the endogenous X. nematophila strain, it had both positive and negative consequences on compound production in X. szentirmaii. Production of

![FIGURE 3](https://example.com/fig3.png)
GameXPeptide C, the main GameXPeptide derivative in *X. szentirmaii*, is increased sixfold while xenofuranone (Brachmann et al., 2006) and szentiamide (Ohlendorf et al., 2011) production were reduced to $\sim 0.1 \times$ and $\sim 0.2 \times$ compared to the wild type, respectively (Figure 5). By comparison of the induced and non-induced base peak chromatograms new

| Natural product | ID     | Annotation                                         | wt/lrp |
|-----------------|--------|----------------------------------------------------|--------|
| Unknown         | XNC1_0646 | Non-ribosomal peptide synthetase (NRPS, fragment) | 13.75  |
| Unknown         | XNC1_1561 | NRPS                                              | 14.98  |
| Xenocoumacins   | XNC1_1698 | Desaturase XcnN                                   | 3.43   |
|                 | XNC1_1699 | Dehydrogenase XcnM                                | 8.00   |
|                 | XNC1_1700 | Polyketide synthase (PKS) XcnL                    | 11.15  |
|                 | XNC1_1701 | Non-ribosomal peptide synthase XcnK               | 12.11  |
|                 | XNC1_1702 | Conserved hypothetical protein XcnJ               | 9.62   |
|                 | XNC1_1703 | Thioesterase XcnI                                  | 11.81  |
|                 | XNC1_1704 | PKS XcnH                                           | 13.41  |
|                 | XNC1_1705 | Peptidase XcnG                                     | 11.04  |
|                 | XNC1_1706 | PKS XcnF                                           | 12.05  |
|                 | XNC1_1707 | Acyl-CoA dehydrogenase XcnE                        | 15.66  |
|                 | XNC1_1708 | Putative acyl carrier protein XcnD                 | 14.14  |
|                 | XNC1_1709 | Methoxymalonate biosynthesis protein XcnC          | 18.31  |
|                 | XNC1_1710 | 3-hydroxyacyl-CoA dehydrogenase XcnB               | 17.77  |
|                 | XNC1_1711 | NRPS XcnA                                          | 12.43  |
| Xenotetrapeptide| XNC1_2022 | NRPS XtpS                                         | 2.14   |
| Unknown         | XNC1_2038 | NRPS                                              | 2.15   |
|                 | XNC1_2039 | NRPS                                              | 2.68   |
|                 | XNC1_2040 | NRPS                                              | 2.11   |
| Unknown         | XNC1_2152 | Hypothetical protein                               | 3.67   |
|                 | XNC1_2153 | Arginine aminomutase                               | 29.52  |
|                 | XNC1_2154 | Aminotransferase                                   | 16.99  |
|                 | XNC1_2155 | Putative clavaminate synthase                      | 11.40  |
|                 | XNC1_2156 | PKS                                               | 9.17   |
|                 | XNC1_2157 | PKS/NRPS hybrid                                    | 5.75   |
|                 | XNC1_2158 | Putative membrane protein                          | 5.41   |
|                 | XNC1_2159 | Putative 2-dehydroxy-2-reductase                    | 4.05   |
|                 | XNC1_2160 | Putative dioxygenase                                | 2.88   |
|                 | XNC1_2161 | NRPS                                              | 3.04   |
|                 | XNC1_2162 | Thioesterase (fragment)                            | 2.47   |
| Rhabdopeptides  | XNC1_2228 | NRPS RdpA                                         | 6.37   |
|                 | XNC1_2229 | NRPS RdpB                                         | 8.50   |
|                 | XNC1_2230 | NRPS RdpC                                         | 7.46   |
| Unknown         | XNC1_2233 | NRPS                                              | 2.03   |
| Xenortides      | XNC1_2299 | NRPS XndA                                         | 3.57   |
|                 | XNC1_2300 | NRPS XndB                                         | 4.50   |
| Unknown         | XNC1_2464 | NRPS                                              | 1.98   |
|                 | XNC1_2465 | NRPS                                              | 2.27   |
|                 | XNC1_2466 | NRPS                                              | 2.61   |
|                 | XNC1_2467 | NRPS                                              | 2.89   |
|                 | XNC1_2468 | Putative N-acetyltransferase                       | 3.99   |
|                 | XNC1_2469 | Putative hydroxylase                               | 5.06   |
|                 | XNC1_2470 | Putative aminotransferase                          | 5.67   |
| Xenematides     | XNC1_2713 | NRPS                                              | 5.33   |
| PAX-Peptides    | XNC1_2781 | NRPS XpsB                                         | 8.91   |
|                 | XNC1_2782 | NRPS XpsB                                         | 9.93   |
|                 | XNC1_2783 | NRPS XpsA                                         | 10.14  |
|                 | XNC1_2784 | Putative ABC transporter                           | 10.10  |
| Unknown         | XNC1_2799 | NRPS (fragment)                                    | 0.36   |
FIGURE 4 | FACS analysis detecting YPet (yellow fluorescent protein) fluorescence intensity (per individual cell) of 50,000 events for each condition (A) and HPLC-MS analysis (B) detecting xenematide A (1–4) or xenortide A (5–8). Analyses were performed on X. nematophila HGB081. P_{BAD}-lrp carrying ypet promoter-reporter plasmids for P_Xene (1, 2) or P_Xenor (5, 6) or an empty vector negative control (nc; 3, 4, 7, 8). Strains were either induced (+ara) or not induced by addition of 0.2% arabinose to growth cultures. (1–4) EIC m/z 466.3 [M+H]⁺ for xenematide A. (5–8) EIC m/z 410.3 [M+H]⁺ for xenortide A. Figure 4A was created with FCS Express 5.

FIGURE 5 | Production of selected natural products after heterologous expression of leuO from P. luminescens TTO1 (P) and X. nematophila HGB081 (X) in X. szentirmaii compared to wild type and vector control (vc) as analyzed by HPLC-MS. Cultures supplemented with 0.2% arabinose for P_{BAD} induction are striped and marked with (+) otherwise with (−). (A) Average production of the triplicates (three individual cultures inoculated from the same preculture) was normalized to the appropriate wild type strains without addition of arabinose (−), error bars mark the standard deviation. Values for compounds produced in very low amounts are presented above the corresponding bars. (B) EICs of regulated but structurally unknown compounds.

compounds with m/z 659.3 and 772.3 [M+H]⁺ were identified (Figure 5) that might belong to the family of rhabdopeptides due to their characteristic fragmentation pattern [Reimer et al., 2013; see Supporting Information (Supplementary Figure S1)] as well as m/z 531.3 [M+2H]²⁺ and m/z 630.3 [M+2H]²⁺ which are structurally still unknown. High resolution high performance liquid chromatography coupled with mass spectrometry (HR-HPLC-MS) suggests a sum formula of C_{52}H_{92}N_{11}O_{12} for m/z 531.3 [M+2H]²⁺ indicating a peptide structure.
DISCUSSION

The Role of Lrp, LeuO, and HexA on Natural Product Biosynthesis in Entomopathogenic Bacteria

Understanding the regulation of natural product biosynthesis is an important step toward predicting the function of natural products if it is known under which conditions the regulators are active. The regulatory cascades controlling natural product biosynthesis have not been investigated in detail in entomopathogenic bacteria. The global regulator HexA has already been the focus of several studies on *Photobacterium denitrificans* and was found to play an important role as a repressor in the nematode host. It has been reported that it has an impact on IPS and anthraquinone biosynthesis (Kontnik et al., 2010) and that HexA itself is regulated by another global regulator. A *A. hadar* mutant hardly produces secondary metabolites while an additional deletion of *hexA* restores the production (Tobias et al., 2016). We wanted to enhance these studies with the focus on additional compounds directly comparing *hxA* inducing and non-inducing conditions. Indeed, we observed that HexA generally acts as a repressor for all compounds that have been analyzed in *P. luminescens*. Lrp as a global transcriptional regulator has been reported in both *Photobacterium denitrificans* and *Xenorhabdus nematophila*, but not directly upon natural product BGCs (Cowles et al., 2007; Kontnik et al., 2010; Lango-Scholey et al., 2013; Hussa et al., 2015). The greatest influence of *P. luminescens* TTO1 Lrp on a known compound is the overproduction of desmethylphurealipid A (∼4×) while LeuO has very little effect (Figure 2). Overproduction (∼5×) of this compound was also observed in the non-induced PBAD-*hxA* strain. In vivo experiments in which this compound and other phurealipids were injected into *Galleria mellonella* and *Manduca sexta* larvae showed reduced levels of mRNA for insect antimicrobial-peptide-encoding genes suggesting a role for these molecules in insect pathogenicity (Nollmann et al., 2016). In contrast, the non-induced Lrp and HexA mutant both produce significantly more mevalagmapeptide than the wild type (∼4× and ∼5×, respectively) or the induced mutant. The role of mevalagmapeptides for the producing organism has yet to be established. Lrp has previously been reported to have either no significant (Kontnik et al., 2010), as well as activating effects (Lango-Scholey et al., 2013), on IPS biosynthesis. Lango-Scholey et al. (2013) suggested that this discrepancy might be due to small differences in the corresponding lab strains. In our study, we found little influence of Lrp on stilbene production. However, we saw activation by LeuO on its biosynthesis, while HexA represses it. If *hxA* is not induced, IPS levels are ∼6× above wild type level, which supports previous studies (Kontnik et al., 2010). We suggest that other factors such as nutrients might play an additional role for Lrp in regulating IPS biosynthesis, as we also observed an increase in IPS production for the induced strain when LB was used for cultivation instead of SF-900 broth (data not shown). For the decreased IPS production in the absence of Lrp observed by Lango-Scholey et al. (2013), the limiting factor appeared to be the branched-chain α-ketoacid dehydrogenase encoded in the *bkdABC* operon that is part of the IPS biosynthesis pathway (Joyce et al., 2008) and is also responsible for the production of branched chain fatty acids (BCFAs) in *P. luminescens* (Lango-Scholey et al., 2013). BCFAs are also part of the photopyrone and phurealipid biosynthesis and their production is also negatively affected by the absence of Lrp. The photopyrones are signal molecules inducing production of the *Photobacterium denitrificans* clumping factor (*pcf*) through a recently described cell–cell communication system (Brachmann et al., 2013). Heterologous expression of the *pcf* operon in *E. coli* greatly enhanced the toxicity toward insect larvae, demonstrating the contribution of photopyrones toward the virulence of *P. luminescens*.

Except for nematophin and xenotetrapeptide, Lrp strongly activates secondary metabolite production in *X. nematophila*, while LeuO attenuates the production of most examined natural products, including nematophin. Nematophin possesses antifungal and antibacterial activities (Li et al., 1997) that might be restricted to *Staphylococcus* strains (Kennedy et al., 2000) although Lang et al. (2008) could neither reproduce these effects, nor could they determine an activity against other bacterial species. Xenocoumacins, xenematides, and xenortides display antimicrobial activity against a broad spectrum of bacteria (Lang et al., 2008) and are thought to eliminate microbial competitors to defend the nutrients within the insect cadaver. Xenematides have insecticidal activity, rhodopeptides are active against insect hemocytes (Lang et al., 2008; Reimer et al., 2013), and xenortides are cytotoxic against mammalian L6 cells (Reimer et al., 2014). The regulation of these compounds by Lrp is in agreement with the previously reported finding that this regulator is needed for virulence (Cowles et al., 2007). Additionally, we show that Lrp affects promoter activity for *P. aeruginosa* and *P. xenorhabdus* (Figure 4A). Consistent with these data, microarray analysis revealed Lrp-dependent regulation of the known biosynthesis genes and clusters for xenematides, xenortides, rhodopeptides, xenocoumacins, and PAX-peptides, as well as six other biosynthetic genes of which the exact function is as yet unknown. Each of these clusters was identified through RNAseq analysis as being positively regulated by the global regulator Fliz in the F1 strain of *X. nematophila* (Jubelin et al., 2013). Since Lrp also positively regulates *fliz* the likely regulatory hierarchy is one in which Lrp positively regulates Fliz, which in turn activates expression of biosynthetic clusters (Hussa et al., 2015). However, members of the Lrp regulon that are either directly or indirectly regulated by Lrp remain to be distinguished. The microarray analysis also shows a slightly higher gene expression for the xenotetrapeptide biosynthesis gene although the relative quantification of this compound revealed a slight downregulation by Lrp. This discrepancy may be explained by differences in growth conditions (LB versus SF-900) or strain identities (ATCC19061 versus AN6/1). One biosynthetic gene, *XNC1_2799* is apparently negatively regulated by Lrp. LeuO in *X. nematophila* acts as a repressor for all examined secondary metabolites except xenotetrapeptide and xenematide A, analogous to its role in *V. cholerae*, *V. parahaemolyticus*, and *S. enterica* in which this regulator attenuates virulence.
(Whitaker et al., 2012; Bina et al., 2013; Espinosa and Casadesús, 2014). This is in contrast to *P. luminescens* TTO1 where LeuO acts as an activator as well as a repressor of secondary metabolism.

As we found that the non-induced P<sub>BAO</sub>-*lrp* mutants produced a significantly lower amount of several compounds but the induced expression of Lrp did not fully restore the production, it is likely that the induced constitutive expression does not reach the natural Lrp levels.

**Heterologous Expression of leuO Reveals Control on Secondary Metabolism in *X. szentirmaii***

Surprisingly, we found that expression of *X. nematophila leuO* in *X. szentirmaii* has a major influence on secondary metabolite expression while *P. luminescens leuO* has no effects (Figure 5). The *X. nematophila* and *X. szentirmaii* LeuO sequences are more similar to each other (90% based on blastx analysis) than either is to *P. luminescens* LeuO (71 and 71%, respectively) indicating that the *P. luminescens* regulator may not be functional in *Xenorhabdus* strains. The impact of LeuO on the synthesis of two natural products is particularly notable when comparing these three organisms. In *P. luminescens* and *X. szentirmaii* GameXPeptides are positively regulated by *leuO* expression. Similarly, rhabdopeptide biosynthesis in *X. nematophila* and *X. szentirmaii* is attenuated by LeuO. The finding that the global regulator LeuO controls the production of the same compound classes indicates that, to some extent, it plays a similar role in the different entomopathogenic *Photorhabdus* and *Xenorhabdus* species.

**Global Regulators as Tools for the Discovery of New Secondary Metabolites and Increased Production of Useful Molecules**

The development of new drugs, especially new antibiotics, is an important research area as resistances against common antibiotics are increasing dramatically (WHO, 24 July 2015).<sup>1</sup> The entomopathogenic *Photorhabdus* and *Xenorhabdus* spp. are a rich source of compounds with antimicrobial bioactivity, but isolation for structural characterization and bioactivity assays is often problematic as the amounts are often low. One method for improving biosynthesis is the exchange of the promotor in front of the biosynthesis gene (Bode et al., 2015) or heterologous expression (Schimming et al., 2014). Unfortunately, these approaches require knowledge about the biosynthetic pathways and the genes necessary for their expression. In the case of heterologous expression another issue is that some biosynthetic precursors might be missing in the heterologous host organism (Nollmann et al., 2015a). One approach that avoids these issues is to elicit overproduction of the molecule(s) in the original producers without knowledge of the relevant biological pathways. Based on the work presented here, the global regulators Lrp and LeuO could be exploited for this approach. For example, overproduction could be achieved by expression of regulators under control of a strong constitutive promoter, or the use of a replicative overexpression plasmid. The potential success of this strategy is supported by our finding that ectopic expression of the *X. nematophila* LeuO regulator in *X. szentirmaii* led to overproduction of the GameXPeptide (Figure 5). Further, this approach can also reveal previously unrecognized compounds, such as the two compounds with *m/z* 531.3 [M+2H]<sup>2+</sup> and *m/z* 630.3 [M+2H]<sup>2+</sup> expressed by *X. szentirmaii* carrying the *X. nematophila* LeuO. The heterologous overexpression of activators like LeuO or Lrp from diverse *Photorhabdus* and *Xenorhabdus* strains offers the possibility to increase the production of specific compounds in strains that have not been sequenced yet.

Although requiring *a priori* knowledge of genome sequence, the inducible production of Lrp in endogenous hosts also has the power to reveal novel compounds. For instance, by comparing base peak chromatograms and the corresponding mass spectra, we identified nine new compounds produced by *P. luminescens* and *X. nematophila*. This discovery might mainly be due to the use of SF-900 broth, a medium that is used for culturing insect cell lines instead of the normally used LB, as most compounds were also present in the wild type and the production levels between wild type and the induced mutant were similar for the individual compounds. Three of these natural products were produced in significantly higher amounts in the induced (*m/z* 321.1 [M+H]<sup>+</sup>) or non-induced (*m/z* 315.0 [M+H]<sup>+</sup>, *m/z* 317.0 [M+H]<sup>+</sup>) *P. luminescens* P<sub>BAO</sub>-*lrp* mutant than in the wild type, suggesting the capacity of this approach to expand the discernible array of secondary metabolites. For comparison of production in SF900 and LB, the base peak chromatograms of *P. luminescens* and *X. nematophila* extracts are displayed in Figure 3K. It is visible that components of LB disturb the chromatogram between retention time (RT) 5–7 min while it is easier to observe new compounds in the cleaner background of SF900 medium.

**CONCLUSION**

We have established that the global regulators Lrp, HexA, and LeuO are involved in the regulation of secondary metabolism in *X. nematophila* and *P. luminescens*, with different effects (activating, repressing, or both) depending on the organism and the individual natural products. A potential for increasing secondary metabolites in heterologous hosts has been shown for *X. nematophila* LeuO in *X. szentirmaii*. The heterologous expression of *lrp* and *leuO* in other organisms remains to be studied but promises to reveal previously unrecognized metabolites.

**AUTHOR CONTRIBUTIONS**

YE performed all experiments except the microarray analysis, which were performed by XL and analyzed by XL and HG-B. The *P. luminescens* and *X. nematophila* promoter exchange mutants
and the X. szentirmaii pBAD30_kanR strains were performed by CW. HB and YE wrote the paper with input from all authors.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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