Genes acquired by horizontal gene transfer share common regulatory proteins in *Photorhabdus laumondii*

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Abstract

Biosynthetic gene clusters are readily transferred between microbes through horizontal gene transfer, but to provide benefit to their host they must be induced to high levels during times of stress. Working with the insect pathogen *Photorhabdus laumondii*, here we study the effect of bacterial enhancer binding proteins (bEBPs) on natural product biosynthesis. We find that the AAA+ ATPase core of the *Sinorhizobium meliloti* bEBP DctD induces expression of five natural product classes in *P. laumondii* TTO1. Similar induction was also observed with truncated copies of bEBPs native to *P. laumondii*, revealing both repression and induction across six natural product classes. Overall, this indicates that bEBPs have the potential to regulate natural product biosynthesis in *P. laumondii*.

Introduction

Driven by advances in genomic sequencing the field of natural product discovery is in a renaissance. Genomic sequencing has shown that even well studied *Streptomyces* strains express less than a quarter of their biosynthetic potential under laboratory conditions (Tanaka *et al.*, 2013). The remaining biosynthetic gene clusters (BGC) are tightly repressed, and the vast majority of compounds they produce are uncharacterized. While industry largely ended their bioprospecting efforts in the 1990's due to the repeated re-isolation of known bacteria and well-characterized natural products (Katz and Baltz, 2016), it appears that the tens of thousands of strains isolated during the golden age of antibiotics are far from exhausted. Improving our understanding of bacterial gene regulation may lead to the discovery of hundreds of thousands of new natural products.

Activating these silent BGC can be a significant undertaking. In many cases the key regulators that control these clusters are unknown, requiring either resource-intensive heterologous expression or combinatorial analysis of growth conditions and possible chemical inducers (the OSMAC approach) (Romano *et al.*, 2018). However, it may be possible to sidestep these issues by taking advantage of the mobile nature of BGC themselves. BGC can be readily transferred between related and unrelated strains via horizontal gene transfer (HGT) (Jensen, 2016), a process that is widespread in terrestrial and marine environments (McDaniel *et al.*, 2010; Aminov, 2011; Ziemert *et al.*, 2014). We hypothesize that to maximize fitness these newly acquired BGC will be transcribed only when they provide the greatest advantage to their new host, and so regulatory elements in the incoming DNA must be recognized by the host machinery. Identification of these regulatory sequences and the transcription factors that bind to them will then allow pan-activation of sequences acquired via HGT.

One such potential regulator was identified following transfer of the BGC for oxytetracycline from *Streptomyces rimosus* ATCC 10970 to the model bacterium *Escherichia coli* BAP1 (Stevens *et al.*, 2013). Overexpression of a single protein, the sigma factor RpoN (σ^{54}), was sufficient to activate the cluster and produce oxytetracycline in *E. coli*. This alternative sigma factor binds to RNA polymerase, determining its promoter specificity (Bush and Dixon, 2012). The consensus sequence for σ54 is well known, and a screen of BGCs in Actinibacteria, Firmicutes and Proteobacteria revealed that ~70% of polyketide and non-ribosomal peptide synthase-

containing clusters contained σ^{54} promoters, despite the passage of billions of years since the last common ancestor between these three *phyla* (Stevens *et al.*, 2013).

However, in Proteobacteria σ^{54} is necessary but not sufficient for expression of genes from σ54 promoters. One of several bacterial enhancer binding proteins (bEBPs) are required to hydrolyze ATP and initiate transcription (Bush and Dixon, 2012). These bEBPs appear to direct activation of σ^{54}-linked genes in response to stress, with roles in phage shock, nitrogen limitation, tyrosine biosynthesis, among others (Bush and Dixon, 2012). Most bEBPs consist of a three interlinked domain, with an N-terminal regulatory domain that activates the protein in response to a cognate kinase or chemical signal, a AAA+ ATPase domain for the initiation of transcription, and a C-terminal DNA binding domain that directs the bEBP to its intended genes. When expressed in isolation the central domain pan-activates σ^{54}, and the core domain of the bEBP DctD from *Sinorhizobium meliloti*, tDctD, has been used to determine the σ^{54} regulon in *S. meliloti* and *Salmonella enterica* (Xu *et al.*, 2004; Samuels *et al.*, 2013).

Interested in the effect of bEBP overexpression on natural product biosynthesis, we have expressed tDctD in the well-characterized entonomopathogen *Photorhabdus laumondii* TTO1. Metabolite production was assessed via LC-MS, revealing activation of a number of BGCs that are poorly expressed during normal lab growth. We then rendered five of the six bEBPs native to *P. laumondii* TTO1 constitutively active through cleavage of their inhibitory N-terminal regulatory domains. Metabolomic analysis of these revealed that the truncated GlrR increased the production of all six natural products under investigation, while TyrR repressed the production of all six.

Materials and Methods

Molecular biology

The strains and plasmids used in this study are listed in Table 1. *E. coli* MFDpir was a generous gift from Luis Ángel Fernández Herrero (Centro Nacional de Biotecnología, Madrid, Spain) (Ferrières *et al.*, 2010), while *P. laumondii* TTO1 was purchased from DSMZ (Braunschweig, Germany). pARO190 was acquired from Cedarlane (Burlington, Canada). The coding sequence for the *Sinorhizobium meliloti* gene $dctD_{141\text{-}394}$, with flanking KpnI and HindIII recognition

sequences, was synthesized as a single gBlock by Gen9 (see supplementary materials), digested, and then ligated into pARO190 with T4 DNA ligase. Genes for each other bEBP were amplified from the *P. laumondii* TTO1 genome (see Table S1 for a list of primers), and similarly digested with KpnI and HindIII prior to ligation into pARO190. As *glrR* contained a KpnI site for this gene HindIII and SaII were used instead. Each plasmid was then transformed into *E. coli* MFDpir cells previously made competent with TSS buffer (Chung and Miller, 1993). The plasmids were then transferred into *Photorhabdus laumondii* TTO1 via conjugation. In brief, cells from both strains were grown overnight in TSB at 30 °C and 225 rpm (with *E. coli* MFDpir cells supplemented with 0.3 mM diaminopimelic acid (DAP) and 100 µg/L of carbenicillin). Cells were then diluted 1:100 into fresh media and grown until an OD_{600} of 0.4. Cells were centrifuged for 1 min at 13 krpm, the supernatant was discarded, and the cells were resuspended in fresh media at their former concentration. *E. coli* and *P. laumondii* were then mixed in a 1:3 ratio, and plated onto TSA plates supplemented with diaminopimelic acid. Following overnight incubation at 30 °C colonies were resuspended in TSB and plated directly onto TSA plates supplemented with 100 µg/L of carbenicillin but not DAP for a period of 48-72h at 30 °C. Repeated attempts to transform *P. laumondii* by chemical or electrical means were unsuccessful.

Identification of putative σ^{54} binding sites

Putative sigma factor binding sites were located with the online Motif Locator (https://www.cmbl.uga.edu/software/motloc.html) **(Mrázek *et al.*, 2008)**. *P. laumondii* TTO1 was selected from the list of previously uploaded genomes, while a list of validated intragenic and intergenic σ^{54} sites from *E. coli* MG1655 were used as input sequences **(Bonocora *et al.*, 2015)**. Trial runs with the genome of *E. coli* MG1655 were used to determine the minimum motif score. A score of 14 best recapitulated the previous *E. coli* experiments (177 hits with Motif Locator vs 135 from ChIP-seq analysis; 75/135 identical in both).

Hypergeometric calculations were performed via the phyper package in R or with an online hypergeometric calculator (https://aetherhub.com/Apps/HyperGeometric). The coding regions of NRPS from the *P. laumondii* genome were added and used as the number of successes in the sample (228,311 of 5,612,110 bases). Results are reported as 1 – the cumulative distributive function, ie. the likelihood of discovering N or greater successes in the sample. Binomial tests were calculated in Excel, assuming a 50% likelihood of sense/antisense orientation.

Metabolomics analysis

An overnight culture of *P. laumondii* containing each plasmid of interest was diluted 1:100 into tryptic soy broth supplemented with 100 µg/L carbenicillin, then incubated at 30 ℃ with shaking at 250 rpm for four days. 0.5 mM IPTG was added to induce bEBP production, while 2% v/v Amberlite XAD16 resin was used to capture produced metabolites. At the end of this incubation the beads and cells were collected by centrifugation at 4 krpm for five minutes, washed three times with Tris-buffered saline (pH 7.6), and then extracted with a 1:1 mixture of methanol:acetone. The solvent was then removed via speed-vac, and the residue was suspended in 1 mL MeOH. Leucine encephalin acetate was added to a final concentration of 1 µM to serve as an internal control. These extracts were then visualized on an Orbitrap LTQ Velos mass spectrometer (Thermo Electron Corporation, San Jose, CA) with a 2.7 µm Cortecs T3 reversed phase column (Waters Corporation, Milford, MA), measuring 2.1x 100 mm. 10 uL of each compound was injected, then separated at a constant flow rate of 0.3 mL/min, using a mixture of phase A (water containing 0.1% formic acid) and phase B (acetonitrile containing 0.1% formic acid) at the following gradient: 5% B for 1 min, a linear increase to 95% B at 20 min, isocratic with 95% B for 7 min, then a linear decrease to 5% B over 7 min. A full MS spectrum (m/z 150-2000) was acquired at a resolution of 100000,

with the five most abundant singly and doubly charged ions were selected each second for fragmentation in a linear trap. Compound fragmentation was performed using a collision induced dissociation at normalized collision energy of 35% ,with an activation time of 10 ms. The spectra were internally calibrated using diisooctyl phthalate (m/z 391.2843 Da) as a lock mass. All data was collected in triplicate, with three biological replicates for each bEBP.

Data analysis

Compound Discoverer 3.3 and Thermo XCalibur Qual Browser were used to analyse the data collected from the LC-MS run. Compounds were selected based on a mass tolerance of 5ppm at MS1 and 0.6 Da at MS2, with compound identities tentatively assigned based on fragmentation patterns.

Results and Discussion

Selection of Photorhabdus as the test strain

Photorhabdus spp. are nematode-associated insect pathogens well-known for natural product biosynthesis (Chaston *et al.*, 2011; Vizcaino *et al.*, 2014; Bozhüyük *et al.*, 2017). While attached to the gut of nematodes *Photorhabdus* spp. are commensal and express few if any natural products (Somvanshi *et al.*, 2012). However, when the nematode infects an insect it will eject its less adhesive bacterial cargo. This variant of *Photorhabdus* spp. produces a wide variety of insecticidal proteins and antibiotics, killing the insect and sanitizing its corpse. This creates a nutrient-rich milieu that is ideal for the growth of both bacteria and nematodes (Chaston *et al.*, 2011). Analysis of publically available *Photorhabdus* genomes by antiSMASH (Weber *et al.*, 2015) revealed two distinct clades (Fig. 1). The first contained four or fewer BGCs, in line with the majority of other sequenced Gammaproteobacteria (Fig. S1). The second clade was far more productive, with on average more than twenty BGCs (Fig. 1). Both clades were present in the three *Photorhabdus* subspecies (*P. asymbiotica; P. luminescens and P. laumondii; P. temperata*), suggesting that the divide is the result of evolutionary pressures and not an artifact of the small number of sequenced strains. As the genomes of *Photorhabdus* spp. rich in BGC also contain a

large number of mobile genetic elements (Ogier *et al.*, 2010), it seems likely that these bacteria have recently acquired a host of BGC to aid in insect pathogenesis. This includes *P. laumondii* TTO1, which contained 21 putative biosynthetic gene clusters as identified by antiSMASH (Table S2).

A pBLAST search of the *P. laumondii* TTO1 genome with the *E. coli* variant of σ^{54} returned one hit with 77% identity and 88% similarity, putatively identified as a multispecies σ^{54}. We then used the online bioinformatics tool motif locator to identify putative σ^{54} binding sites in the *P. laumondii* TTO1 genome (Mrázek *et al.*, 2008; Bonocora *et al.*, 2015) (Tables S3 and S4). A large number of such sites were found, in line with previous analyses of *E. coli* and *Salmonella enterica* genomes (Samuels *et al.*, 2013; Bonocora *et al.*, 2015). 89% of these sites were intragenic (211/236), well above the 62% identified in *E. coli* (Bonocora *et al.*, 2015). Non-ribosomal peptide synthetase (NRPS) BGCs were particularly enriched in putative σ^{54} sites, with 20/236 such sites located within NRPS or NRPS-PKS coding regions, including in clusters known to produce the gameXPeptides and mevalagmapeptides (Tables 2, S3, and S4). This is significantly more than expected from a random distribution (Hypergeometric test $p = 0.00175$), suggesting σ^{54} plays a key role in the regulation of these biosynthetic pathways. As with previous reports we found that far more intragenic σ^{54} sites were in the sense orientation than would be expected due to chance (145/211, Binomial test $p = 2.78 \times 10^{-8}$). However, no similar enrichment was observed in sites within NRPS or NRPS-PKS (12/20, Binomial test $p = 0.251$).

Plasmid construction and metabolomics

To determine if a bacterial enhancer binding protein could alter natural product biosynthesis in *P. laumondii* we inserted the truncated *dctD* gene into pARO190 via standard restriction enzyme digestion and DNA ligation techniques. This plasmid was then transformed into *E. coli* MFDpir, then conjugated into *P. laumondii* TTO1. Cells were then induced with IPTG, and the metabolomes of cells transformed with pARO190 and pARIO190-tdctD were compared. Significant alterations in the LCMS signal of multiple putative natural products were identified (Fig. 2), indicating that this pan-activating bEBP was indeed able to influence natural product biosynthesis. In particular, production of compounds with MS fragmentation patterns consistent with phurealipid A and AQ-270 more than doubled relative to pARO190 (320% and 214%, respectively). Statistically significant increases were also seen in peaks consistent with

photopyrone D (163%), gameXPeptide A (118%), and mevalagmapeptide A (156%). Of the compounds tested, only isopropylstilbene was unaffected (Fig 2).

A pBLAST search of the *P. laumondii* TTO1 genome with the core domain of *dctD* revealed six homologues with an E values less than 1 x 10^{-10} (Table S5). Several of these had significant homology to better-characterized *E. coli* bEBPs, including *glnG, glrR, prpR, pspF,* and *tyrR.* Putative regulatory roles for these bEBPs include nitrogen regulation (*glnG*), propionate catabolism (*prpR*), phage shock (*pspF*), and tyrosine biosynthesis (*tyrR*), among others (Pittard and Davidson, 1991; Shiau *et al.*, 1993; Jovanovic *et al.*, 1996; Lee *et al.*, 2005; Reichenbach *et al.*, 2009). Of these five regulators, four are known to bind to σ54 in *E. coli*, while TyrR, despite its homology, instead interacts with σ^{70} (Camakaris *et al.*, 2021). The last bEBP in *P. laumondii* had no significant homology to any *E. coli* bEBPs, but did contain a PAS regulatory domain. These domains have been previously found to bind to small molecules, indicating a potential chemosensory role (Liu *et al.*, 2015). To determine which, if any, of the native *P. laumondii* bEBPs regulated natural product biosynthesis we then set out to create constitutively active variants of these proteins.

The domain structure of most bEBPs is well understood, with a central ATPase domain flanked on one side by a DNA recognition element, which binds to DNA 60-300 bp upstream of the σ^{54} promoter (Bush and Dixon, 2012). The other end of the bEBP generally contains a regulatory region, which ties bEBP activity to either a cognate kinase or the presence of a chemical signal (for example, NO for the NorR receptor) (Bush and Dixon, 2012). Truncation of the regulatory and DNA-binding regions can create constitutively active bEBPs like tDctD (Xu *et al.*, 2004), while truncation of just the regulatory domain should in principle render the bEBP constitutively active but targeted only to its native genes. As the precise borders of the regulatory domains were not known, we prepared a number of truncated proteins, with cuts either at the end of the regulatory domain (as listed in the UniProt reference sequence), the beginning of the AAA+ ATPase domain, or midway between. In *E. coli* PspF is regulated by binding to an inhibitory protein, PspA, and lacks a regulatory domain. The *P. laumondii* homologue further had a mutated start codon, and so to address both issues we expressed it without truncation, with the start codon mutated to methionine. This allowed us to prepare five of the six *P. laumondii* bEBPs (Table 2),

with four of these successfully conjugated into *P. laumondii*. Repeated attempts to conjugate pARO190-prpR$_{(\Delta 2\text{-}208)}$ resulted in no colonies, indicating this bEBP may be toxic to *P. laumondii*.

As with tDctD, expression of the bEBPs drastically altered natural product biosynthesis in *P. laumondii*. Expression of a truncated copy of GlrR, GlrR$_{(\Delta 2\text{-}122)}$, activated all six of the natural products under investigation, with an average increase in signal of 190% relative to pARO190 alone. The PAS-containing bEBP displayed differential activity based on its truncation point, with the variant truncated midway between the PAS and ATPase domains, PAS$_{(\Delta 2\text{-}304)}$, broadly inactivating the compounds under investigation and the variant truncated to the beginning of the ATPase domain activating five of the six compounds. While more work will be required to determine why PAS$_{(\Delta 2\text{-}304)}$ suppresses natural product production, this behaviour would be expected if an inactive ATPase domain were to bind to and prevent the activation of σ^{54}. TyrR$_{(\Delta 2\text{-}123)}$ also generally suppressed natural product production, though like PAS$_{(\Delta 2\text{-}304)}$ it had little effect on the production of gameXPeptide A. Further truncation of TyrR led to TyrR$_{(\Delta 2\text{-}200)}$. This protein suppressed production of all six natural below detectable limits, with cells forming exclusively small, yellow colonies when grown on agar. These features are consistent with the mutualistic form of *P. laumondii*, suggesting that TyrR may play a role in growth of the bacteria when in its nematodal host (Somvanshi *et al.*, 2012).

Conclusion

Natural product discovery is limited by an inability to effectively control *in situ* natural product biosynthesis. Working with a pan-activating enhancer binding protein from *S. meliloti*, tDctD, we induced the production of a number of known metabolites in *P. laumondii* TTO1. Overexpression of truncated native *P. laumondii* bEBPs similarly altered compound production, with differential activity depending on the bEBP and how much of the proteins' regulatory sequences had been cleaved. Combined, this work provides further evidence that σ^{54} broadly regulates natural product biosynthesis in bacteria, suggesting a common means of activating the tens of thousands of silent clusters found in Gram-negative and Gram-positive bacteria currently stored in strain libraries across the globe.

Author Contributions

LR cloned the pARO190-bEBP plasmids and carried out the LCMS analysis. MA conducted early experiments with tdctD. BF conceived of and designed the experiments, supervised the work, and performed the bioinformatics analysis.

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Additional Information

Supplementary information accompanies this paper.

Competing financial interests: The authors declare no competing interests.

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Figures and Tables

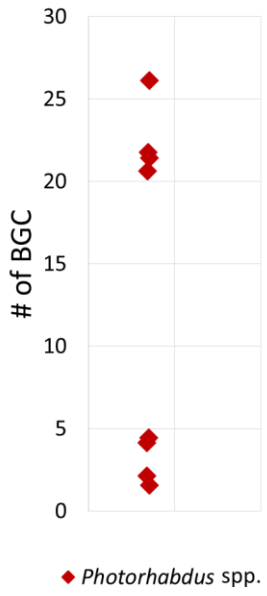

Figure 1. Number of biosynthetic gene clusters within *Photorhabdus* spp. This subset of publically available strains reveals two distinct *Photorhabdus* clades: those with a large number of biosynthetic gene clusters and those with significantly fewer. Genomes were obtained from NCBI and analyzed via antiSMASH 3.0. For a full list of the strains investigated see Table S6.

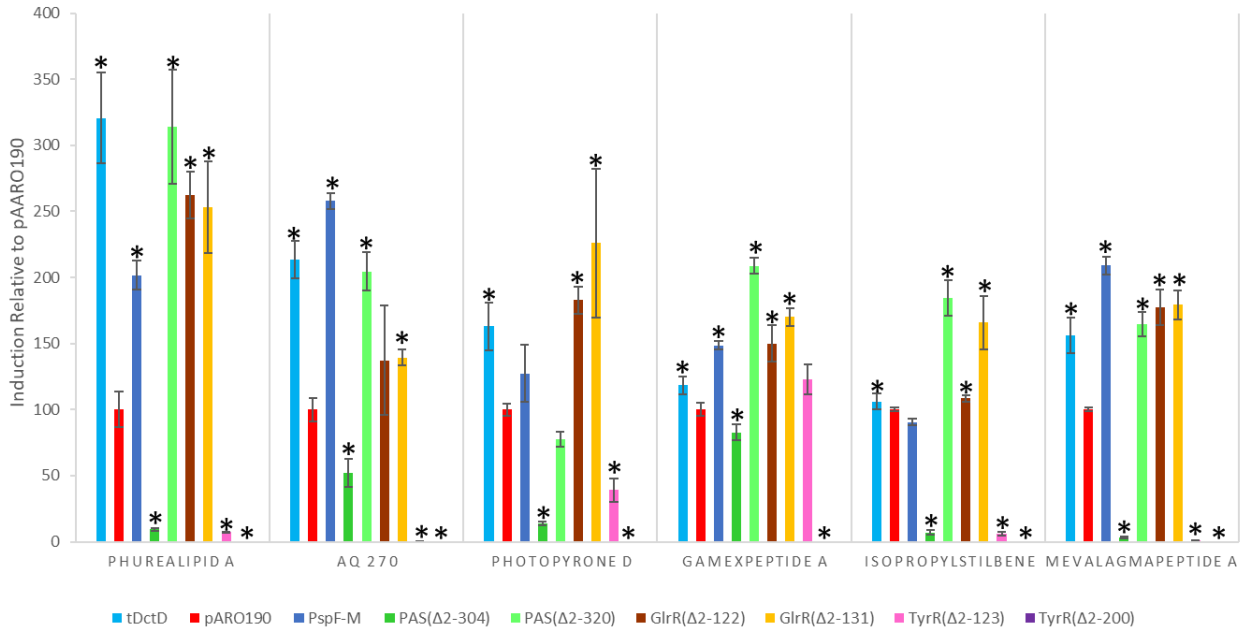

Figure 2. Metabolome following induction. Production of putative *P. laumondii* metabolites was scaled to pARO190. Intensities gathered by LCMS (positive mode), with compounds identities tentatively assigned based on MS/MS analysis. Stars indicate statistically significant variation relative to pARO190 ($p < 0.05$, two-tailed student T test).

Table 1. Strains and plasmids used in this study.

Strain	**Notes**
Photorhabdus luminescens TTO1 (DSM15139)	Wild type, formerly known as *Photorhabdus luminescens* subsp. *laumondii* TTO1.
E. coli MFD(*pir*+)	MG1655 RP4-2-Tc::[ΔMu1::aac(3)IV-ΔaphA-Δnic35-ΔMu2::zeo] ΔdapA::(erm-pir) ΔrecA
E. coli DH5*α*	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ^-. For routine cloning.
Plasmid	**Notes**
pARO190	Amp^r; *bom*, basis of mobilization gens; oriT from RP4. *lacZα*, for blue-white screening (from ATCC)
pARO190-t*dctD*	Contains the AAA+ ATPase core (residues 141-394) from *S. meliloti*
pARO190-*pspF-M*	1st codon of modified from LEU (TTG) to MET (ATG).
pARO190-*PAS*$_{(\Delta 2\text{-}304)}$	
pARO190-*PAS*$_{(\Delta 2\text{-}320)}$	
pARO190-*GlrR*$_{(\Delta 2\text{-}122)}$	
pARO190-*GlrR*$_{(\Delta 2\text{-}131)}$	
pARO190-*tyrR*$_{(\Delta 2\text{-}123)}$	
pARO190-*tyrR*$_{(\Delta 2\text{-}200)}$	
pARO190-*prpR*$_{(\Delta 2\text{-}208)}$	Could not be conjugated into *P. laumondii* TTO1.

Table 2. σ^{54} binding sites in *Photorhabdus laumondii* TTO1 which lie within NRPS or NRPS-PKS.

BGC	**Position**	**Gene**	**Orientation**	**Distance from gene start**	**Distance from gene end**	**Products**	**Reference**

5	1026921	plu0898	Antisense	2924	1793	Mevalagmapeptides	(Wang *et al.*, 2019)
6	1280255	plu1113	Sense	2985	11962	Unknown	
6	1283423	plu1113	Sense	6153	8794	Unknown	
6	1286636	plu1113	Sense	9366	5581	Unknown	
6	1294572	plu1115	Sense	945	4414	Unknown	
7	1413798	plu1220	Sense	3108	69	Unknown	
8	2223806	plu1878	Antisense	1416	1778	Luminmycin A, gliobactin A, cepafungin	(Bian *et al.*, 2012; Wang *et al.*, 2019)
8	2235424	plu1880	Sense	8908	3558	Luminmycin A, gliobactin A, cepafungin	(Bian *et al.*, 2012; Wang *et al.*, 2019)
10	2724351	plu2320	Sense	4076	2068	Piscibactins	(Wang *et al.*, 2019)
10	2724766	plu2320	Antisense	1653	4491	Piscibactins	(Wang *et al.*, 2019)
10	2725347	plu2320	Sense	5072	1072	Piscibactins	(Wang *et al.*, 2019)
10	2732439	plu2321	Antisense	5706	6004	Piscibactins	(Wang *et al.*, 2019)
12	3143522	plu2670	Antisense	30148	19017	Kolossin	(Bode *et al.*, 2015)
12	3144847	plu2670	Antisense	28823	20342	Kolossin	(Bode *et al.*, 2015)
12	3148383	plu2670	Sense	23878	25287	Kolossin	(Bode *et al.*, 2015)
12	3157460	plu2670	Antisense	16210	32955	Kolossin	(Bode *et al.*, 2015)
14	3683221	plu3130	Sense	4693	5241	Unknown	

15	3873015	plu3263	Sense	7923	7759	GameXPeptides	(Nollmann *et al.*, 2015)
17	4147924	plu3534	Antisense	7149	2647	Unknown	
17	4156839	plu3535	Sense	1750	8091	Unknown	

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