Symbiosis, virulence and natural-product biosynthesis in entomopathogenic bacteria are regulated by a small RNA

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***Photorhabdus* and *Xenorhabdus* species have mutualistic associations with nematodes and an entomopathogenic stage^{1,2} in their life cycles. In both stages, numerous specialized metabolites are produced that have roles in symbiosis and virulence^{3,4}. Although regulators have been implicated in the regulation of these specialized metabolites^{3,4}, how small regulatory RNAs (sRNAs) are involved in this process is not clear. Here, we show that the Hfq-dependent sRNA, ArcZ, is required for specialized metabolite production in *Photorhabdus* and *Xenorhabdus*. We discovered that ArcZ directly base-pairs with the mRNA encoding HexA, which represses the expression of specialized metabolite gene clusters. In addition to specialized metabolite genes, we show that the ArcZ regulon affects approximately 15% of all transcripts in *Photorhabdus* and *Xenorhabdus*. Thus, the ArcZ sRNA is crucial for specialized metabolite production in *Photorhabdus* and *Xenorhabdus* species and could become a useful tool for metabolic engineering and identification of commercially relevant natural products.**

Regulation via *trans*-encoded small RNAs (sRNAs) typically occurs by imperfect base-pairing of sRNAs with their messenger-RNA targets and can be mediated by RNA chaperones such as Hfq and ProQ^{5,6}. RNA duplexes are usually short (6–12 nucleotides) and can result in conformational changes in the RNA secondary structure with various regulatory outcomes⁷. The RNA chaperone Hfq is highly conserved throughout the bacterial kingdom⁸. Several complex phenotypes have been attributed to Hfq, which performs its regulatory roles by stabilizing sRNAs and/or mRNAs, mediating base-pairing of sRNAs and their targets, modulation of mRNA translation⁸ as well as accelerating the degradation of sRNAs and their targets⁵. The expression of sRNAs is highly dynamic, with sRNA profiles in *Salmonella* shown to be strongly dependent on the bacterial growth phase⁹. ArcZ is one of the few Hfq-bound sRNAs whose expression remains relatively constant in *Salmonella* throughout the growth phases, constituting approximately 7–12% of all reads identified by Hfq co-immunoprecipitation experiments⁹. ArcZ is transcribed as a 129-nucleotide primary transcript (Fig. 1a) and processed into a

stable short form (approximately 50 nucleotides)^{9–11}. The processed short form of ArcZ activates *rpoS* translation directly and inhibits the expression of several other genes^{11,12}. In *Escherichia coli*, the expression of *arcZ* is repressed by the ArcA–ArcB two-component system under anaerobic conditions. In a negative feedback loop, *arcZ* represses and is repressed by *arcB* transcription¹¹. Although there is a wealth of research on ArcZ in *E. coli* and *Salmonella*^{9–11}, its function in other bacteria remains unclear.

Specialized metabolites (SMs) in bacteria are often responsible for ecologically important activities¹³. In the case of *Xenorhabdus* and *Photorhabdus*, SMs play an essential role in cross-kingdom interactions with nematodes, various insects as well as bacterial and fungal species competing for the same food source¹⁴. Our earlier work on *Photorhabdus* showed that the deletion of *hfq* results in severe perturbation of gene networks, including several key regulators⁴. This leads to an overall decrease in SM production and a failure of the bacteria to support their obligate symbiosis with nematodes. Despite SMs playing a central role in the life cycle of the symbiosis, the exact ecological functions of many of these compounds has remained unknown. Notable advances towards the identification of the bioactivities of many of the SMs—with assigned functions including cell–cell communication (photopyrones and dialkylresorcinols^{15,16}), nematode development (isopropylstilbene¹⁷), defence against food competitors (isopropylstilbene and rhabdopeptides^{17,18}) or insect pathogenicity (rhabduscin, rhabdopeptides and glidobactin^{18–20})—have been made in recent years. However, understanding the full potential of SMs in these bacteria is still hampered by a somewhat limited understanding of when individual SMs are produced and their regulation in general. The regulators Hfq, HexA (also LrhA), LeuO and Lrp have so far been implicated in the regulation of SMs in *Photorhabdus* and *Xenorhabdus*^{3,4,21,22}. Deletion of *hfq* in *Photorhabdus* results in complex regulatory changes, including a strong upregulation of HexA, a known repressor of SM production⁴. Consequently, SM production is completely abolished in this strain and nematode development was severely restricted.

Given the overlapping life cycles and niche occupation, we hypothesized that the deletion of *hfq* in *Xenorhabdus* would have

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Fig. 1 | ArcZ is a conserved Hfq-binding sRNA. a, Alignment of *arcZ* sequences from *P. laumondii* TTO1, *Photobacterium temperata*, *Photobacterium asymbiotica*, *Xenorhabdus nematophila*, *Xenorhabdus bovienii*, *X. szentirmai*, *Serratia marcescens*, *Erwinia amylovora*, *E. coli* K12 and *Salmonella typhimurium* LT2. The numbers refer to the nucleotide positions of the *P. laumondii* sequence. The +1 indicates the transcriptional start of the 129-nucleotide *arcZ* sequence. The start codon for *elbB* and stop codon for *arcB*, -10 and -35 binding regions as well as the conserved ArcA binding region¹¹ and the region of base-pairing to *hexA* are indicated. The site of ArcZ cleavage is indicated by an arrow. **b**, Enrichment, determined by RIP-seq, in regions of sRNAs. **c**, Comparison of the mRNAs in a strain containing Hfq^{3xFLAG} and the untagged control strain at both optical densities. Yer, yersiniabactin-related genes. For a complete list of the enriched regions see Supplementary Tables 4 and 5. The blue dots represent SM-related mRNAs and orange dots represent mRNAs associated with annotated regulators.

a similar effect on the production of SMs and the transcriptome. We confirmed this in *Xenorhabdus szentirmai* DSM16338 using both high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) and RNA sequencing (Supplementary Note 1 and Supplementary Table 1). We investigated the Hfq binding partners to further elucidate the mechanism of SM regulation. To this end, we sequenced both *X. szentirmai* and *Photobacterium laumondii* using a sRNA sequencing protocol and combined this with Cappable-seq data to globally annotate the transcriptional start sites belonging to coding sequences or potential previously undescribed sRNAs (Supplementary Note 2 and Supplementary Table 2). We confirmed the expression of several

of these sRNAs by northern blot analysis (Extended Data Figs. 1 and 2). To identify RNA-protein interactions on a global scale, we next employed RNA immunoprecipitation, followed by high-throughput sequencing (RIP-seq) using chromosomally produced Hfq^{3xFLAG} protein as bait. We performed these experiments at two different cell densities (that is, at an optical density (OD)₆₀₀ of 0.5 and 5.0; see Supplementary Table 3 for a full list of the ODs from different experiments). From the corresponding sequencing data, we first identified regions of 5 base pairs (bp) or more that were enriched in our tagged Hfq strain (Methods). We then searched for sRNAs that were specifically enriched in the tagged samples compared with the untagged samples. We identified a total of 37 binding

sites in annotated sRNAs (35 unique sRNAs) at an OD₆₀₀ of 0.5 and 37 binding sites (34 unique) at an OD₆₀₀ of 5.0 that were enriched by at least threefold in both replicates (Fig. 1b and Supplementary Table 4). During early exponential growth, 11 sRNAs (of 35) that have been described to associate with Hfq in other species were identified, whereas 10 (of 34) are known from those that were enriched at an OD₆₀₀ of 5.0. As a second step, we examined the mRNAs enriched in the data. At an OD₆₀₀ of 5.0, 402 mRNAs and 32 annotated 5' untranslated regions (UTRs) were identified to associate with Hfq. A total of 1,003 mRNAs and 29 5' UTRs were detected at an OD₆₀₀ of 0.5, (Fig. 1c and Supplementary Table 5).

We hypothesized that the Hfq RIP-seq analysis performed would allow us to identify key sRNAs involved in SM repression. However, our analysis identified >50 potential Hfq-binding sRNAs (across both ODs; Fig. 1b). We therefore constructed a transposon-mutant library using pSAM-BT_Kan instead of deleting each sRNA individually (Methods and Supplementary Note 3) and searched for phenotypes consistent with that of the strain from which *hfq* had been deleted (Δhfq). The red colour afforded to the bacteria by anthraquinone production makes the strain especially suitable for transposon mutagenesis when screening for mutants defective in SM biosynthesis. We screened approximately 60,000 clones for obvious phenotypic alterations. Several mutants were defective in some facets of SM production and showed growth defects (Supplementary Note 3, Extended Data Fig. 3 and Supplementary Table 6); however, only one displayed the desired phenotype. Re-sequencing of this strain, followed by read mapping revealed that the transposon was inserted within an intergenic region associated with the *arcZ* sRNA gene (Supplementary Fig. 1).

ArcZ is a well-known Hfq-associated sRNA, which was also present in our list of Hfq-bound sRNAs in *P. laumondii* (Fig. 1b and Supplementary Table 4). To verify that the observed phenotype was derived from the transposon insertion, we generated a $\Delta arcZ$ mutant by deleting the major part of the sRNA (Supplementary Fig. 1) and a complemented strain by reintroducing an intact version of *arcZ* at the original locus. Northern blot analysis was performed to verify the absence of ArcZ in the deletion mutant and the presence of ArcZ in the WT and the complementation mutant (Fig. 2a). Sequencing of the $\Delta arcZ$ -mutant RNA showed severe transcriptomic changes compared with the WT and $\Delta arcZ::arcZ$ mutant of *P. laumondii*, reminiscent of that seen in *P. laumondii* Δhfq (Supplementary Note 4, Supplementary Tables 7,8 and Supplementary Fig. 2). The SM production titres in the $\Delta arcZ$ strain were strongly decreased, similar to that seen in the transposon-insertion mutant, and the complementation strain restored SM production (Fig. 2c–h).

To corroborate the role of ArcZ in SM production, the ArcZ mRNA targets were predicted using CopraRNA²³ (Supplementary Tables 9 and 10). One hit warranting further investigation was *hexA* (*lrhA*), which was previously identified as a gene that is highly upregulated in our strains and represses SM production in both *P. laumondii*²⁴ and *Xenorhabdus*³. CopraRNA predicted a 9-bp-long RNA duplex involving the 5' UTR of *hexA* and the processed isoform of ArcZ (Fig. 3a). This base-pairing is reminiscent of previously reported ArcZ targets in other bacteria requiring the RNase E-mediated release of the seed region of the sRNA¹². We also identified a corresponding enriched RNA sequence upstream of the *hexA* coding sequence at an OD₆₀₀ of 0.5 in the RIP-seq experiments (Fig. 1c and Supplementary Fig. 3). We hypothesized that, through Hfq, ArcZ might bind to the *hexA* transcript leading to repression of HexA, and that Hfq and ArcZ prevent HexA production in laboratory cultures in which SMs are produced, thereby allowing the strain to synthesize SM. However, we expected that *hexA* would no longer be repressed if either *hfq* or *arcZ* were deleted, resulting in severely reduced SM production. To test this idea, we altered the predicted site of the ArcZ–*hexA* interaction to a PacI restriction site (TTAATTAA) and created a knock-in of *hexA* with the modified

sequence in a $\Delta hexA$ strain (Supplementary Fig. 4a,b). We predicted that the knock-in of *hexA* with an altered 5' UTR would result in a failure of ArcZ to bind, leading to a reduced SM titre. The SM-production titres of the knock-in mutant with the altered binding site upstream of *hexA* were indeed greatly reduced (Fig. 2c–h).

To verify the proposed interaction region, we conducted a compensatory base-mutation study in *E. coli*. The fifth base pair of the proposed interaction region was exchanged in the *arcZ* sequence, the *hexA* 5' UTR or both using site-directed mutagenesis (Fig. 3a). The *hexA* 5' UTR sequence was fused to the green fluorescent protein (*gfp*) gene. The GFP output was measured to determine the efficiency of inhibition (Fig. 3b,c). For the control, the GFP signal derived from the expression of *hexA::gfp* was measured and set to one. When p-*arcZ* was expressed together with *hexA::gfp*, HexA repression was increased 32-fold compared with the control. In addition, when p-*arcZ** (G79C) was expressed, ArcZ* was no longer able to repress HexA. HexA repression was only slightly increased compared with the control for *hexA*::gfp* (C46G) in combination with the native ArcZ, suggesting that ArcZ can still bind to the 5' UTR of *hexA* but with substantially reduced efficiency. When p-*arcZ** (G79C) was combined with *hexA*::gfp* (C46G), HexA::GFP repression was increased 39-fold, which confirms our hypothesis that ArcZ binds to the 5' UTR of *hexA* to repress HexA production. Notably, this base-pairing sequence is located approximately 50 nucleotides upstream of the *hexA* translational start site (Fig. 3a) and thus ArcZ binding is unlikely to compete with recognition of the mRNA by 30S ribosomes²⁵. Instead, alignment of the *P. laumondii* *hexA* 5' UTR revealed that the ArcZ binding site is CA-rich and highly conserved among other SM-producing bacteria (Supplementary Fig. 5). CA-rich sequences located in proximity to translation initiation sites are well-known translational enhancers and sequestration of these regulatory elements by sRNAs has been reported to downregulate gene expression^{26,27}, which might also be relevant for the ArcZ–*hexA* interaction reported here. In addition, we conducted a proteomic analysis with the wild-type (WT), $\Delta arcZ$, Δhfq and $\Delta hexA::hexA_PacI_UTR$ strains of *P. laumondii*. We used label-free quantification of quadruplicate samples to determine the HexA abundance in each strain. The levels of HexA were substantially elevated in all mutant strains (11.8- to 22.7-fold; Supplementary Table 11) compared with the WT, further supporting this mechanism of regulation for SM production.

The *arcZ* gene and its genomic organization are highly conserved among enterobacterial species¹⁰ (Fig. 1a). As the control of SMs in *Photorhabdus* relays a fundamental ability for these bacteria to occupy their specific niche, we investigated the possibility that the same mechanism occurs in the closely related *Xenorhabdus*. Given the SM reduction in *X. szentirmaii* Δhfq , we constructed a $\Delta arcZ$ mutant in *X. szentirmaii* in a similar fashion to *P. laumondii* by deleting 90 bp of the predicted *arcZ* sequence. Using northern blots, we verified that ArcZ was no longer produced by the deletion mutant and that complementation of the deletion led to resumed production of ArcZ (Fig. 3d). We subsequently investigated the transcriptome and SM profile of the WT as well as the deletion and complementation mutants (Fig. 3e and Supplementary Table 12). Consistent with *P. laumondii*, deletion of *arcZ* resulted in a global effect on the transcriptome as well as severe reductions in the SM titre, both of which were complemented in the $\Delta arcZ::arcZ$ complementation mutant (Figs. 3e and 4).

Our results highlight the critical role of ArcZ in regulating specialized metabolism in these strains. In fact, the critical nature of the SMs from *Photorhabdus* and *Xenorhabdus* in modulating the insect immune response indicated that ArcZ might be required for niche occupation by these bacteria. We observed an inability of the *P. laumondii* $\Delta arcZ$ strain to support nematode development (Extended Data Fig. 4), consistent with our earlier observations for the Δhfq mutant⁴. However, the same was not seen with *X. szentirmaii*.

Fig. 2 | Specialized metabolite production in *P. laumondii* mutant strains. **a**, ArcZ expression in *P. laumondii* WT, $\Delta arcZ$ and $\Delta arcZ::arcZ$ cells, determined using northern blot analysis. Total RNA samples were collected at three different OD₆₀₀ values (0.5, 2 and 4) and after 24 h of growth. Probing for 5S ribosomal RNA served as a loading control. The blot image is representative of two biologically independent replicates. M, molecular weight markers. **b**, Comparison of the relative production titres of the major SMs produced by the *P. laumondii* WT, $\Delta arcZ$ and Δhfq strains. The extracted ion chromatograms of anthraquinone (AQ-270a), isopropylstilbene (IPS), phurealipid A (PL-A), GXP-A and photopyrone D (PPY-D) in the WT and mutant strains are depicted. **c-h**, HPLC-MS/MS quantification of AQ-270a (**c**), IPS (**d**), GXP-A (**e**), PPY-D (**f**), PL-A (**g**) and mevalagmapptide (MVAP; **h**) in *P. laumondii* WT, TN::arcZ, $\Delta arcZ$, $\Delta arcZ::arcZ$, $\Delta hexA$, $\Delta hexA::hexA_Pacl$ and $\Delta hexA::hexA$. All bars represent the relative production in comparison to the WT. Data are presented as the mean $\pm$ s.e.m. The dots represent biologically independent replicates ($n = 3$). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ and **** $P < 0.0001$. Statistical significance of the relative production compared with the WT production levels was calculated using a two-sided unpaired *t*-test. The exact *P* values were as follows (from left to right): **c**, <0.0001, not determined (n.d.), 0.2, <0.0001, <0.0001 and 0.24. **d**, n.d., n.d., 0.24, 0.0005, <0.0001 and 0.12. **e**, <0.0001, n.d., 0.18, 0.0018, <0.0001 and 0.0037. **f**, <0.0001, n.d., 0.56, 0.0003, <0.0001 and 0.013. **g**, <0.0001, n.d., 0.0001, <0.0001, <0.0001 and 0.059. **h**, 0.0006, 0.0008, 0.34, <0.0001, 0.011 and 0.0058. Details of all analysed compounds can be found in Supplementary Table 16.

Nearly all of the SM-related genes analysed in both *Xenorhabdus* and *Photorhabdus* were found to be downregulated in the $\Delta arcZ$ mutant, in accordance with impaired SM production (Fig. 4a,b). This provides a chemical background that is devoid of natural products, which allows for the isolation and identification of a desired compound due to the absence of compounds with similar retention

Fig. 4 | Secondary metabolites are regulated by *arcZ* and *hfq* regulons. **a, b**, Comparison of the ArcZ and Hfq regulons in *P. laumondii* (**a**) and *X. szentirmaii* (**b**). The scatterplots show individual coding sequences and their corresponding regulatory changes compared with the WT in either the $\Delta arcZ$ (x axis) or Δhfq (y axis) mutants, with the SMs (blue dots) and regulators (orange dots) highlighted. Inset: only SM-related coding sequences are shown, including those associated with anthraquinone (AQ), mevalagmapptide (MVAP), carbapenem (Cbp), yersiniabactin (Yer), GXP, siderophore (Sid), isopropylstilbene (IPS), glidobactin (Gdb), phenazine (PHZ), fabclavine (FCL), xenoamicin (XA) and pyrrolizinenamide (PXA). **c**, Base peak chromatograms of *X. szentirmaii* WT, $\Delta arcZ::pCEP_GxpS$ (uninduced and induced) and $\Delta hfq::pCEP_GxpS$ (uninduced and induced) strains. Peaks correspond to (cyclo)tetrahydroxybutyrate (THB)⁵⁸, linear GXP-C, rhabdopeptide 772 (RXP 772), xenofuranone A (XF-A), as well as cyclic GXP-C and GXP-D. A $\times 5$ zoom was applied to the base peak chromatograms in the uninduced and WT samples.

times. Therefore, $\Delta arcZ$ mutants could offer a powerful tool for the (over-)production and identification of previously undescribed natural products. As a proof of concept, we conducted a promoter exchange in front of *gxpS* in both *X. szentirmaii* $\Delta arcZ$ and *X. szentirmaii* Δhfq , and compared GameXPeptide (GXP)-C production after induction to the WT (Fig. 4c). The production of GXP-C was found to be increased 90.4-fold (± 4.7) in *X. szentirmaii* $\Delta arcZ::pCEP_GxpS$ and increased 138.6-fold (± 17.1) in *X. szentirmaii* $\Delta hfq::pCEP_GxpS$ compared with the WT (Fig. 4c). The striking increase in production as well as the dramatically reduced chemical background in both strains highlights the potential for exploiting this regulatory cascade for selective SM production in a strain that is well-suited for natural product detection. We recently showed that this strategy could be applied in a high-throughput manner for rapid screening of bioactivities³³. The same strategy used here in a $\Delta arcZ$ strain demonstrates an alternative route to activation, without the complex perturbations associated with deleting the major RNA chaperone in these bacteria. Interestingly, some comparisons between these mechanisms can be drawn in other SM-producing Enterobacteriaceae (Fig. 1a). *Erwinia* is a genus of plant-pathogenic bacteria that produce SMs, where Hfq and ArcZ have both been implicated in virulence³⁴, whereas HexA is a negative regulator of secondary metabolites in these bacteria³⁵. Similar parallels can also be seen in *Serratia*^{36–38} and *Pseudomonas*³⁹, two

other prolific SM producers. Although further investigations will be required to ascertain whether these apparent similarities represent identical mechanisms, the conserved nature of ArcZ in other SM-producing Enterobacteriaceae could suggest that this strategy may yield fresh avenues for rapid investigation into SM biosynthesis in other taxa.

Methods

Bacterial culture conditions. All *Photobacterium* and *Xenorhabdus* strains were cultured in Luria–Bertani (LB) medium for at least 16 h with shaking at 30 °C. The *E. coli* strains were cultured in LB for at least 16 h with shaking at 37 °C. The medium was supplemented with chloramphenicol (34 $\mu\text{g ml}^{-1}$), ampicillin (100 $\mu\text{g ml}^{-1}$), rifampicin (50 $\mu\text{g ml}^{-1}$) or kanamycin (50 $\mu\text{g ml}^{-1}$) when appropriate. Promoter-exchange mutants were induced by adding L-arabinose (2%, vol/vol) to the cultures. All of the plasmids and strains used in this study are listed in Supplementary Tables 13 and 14.

Nematode bioassays. All nematodes were cultivated in *Galleria mellonella* and collected on white traps as previously described⁴⁰. The nematode bioassays were also performed as described elsewhere⁴.

Creation of a transposon-mutant library. For the transposon mutagenesis, the plasmid pSAM_Kan (containing the mariner transposon) was constructed using pSAM_BT⁴¹ as a template. To do this, the plasmid was linearized using the primers NN191 and NN192. The kanamycin-resistance cassette was amplified from the pCOLA_ara-tacI plasmid using the primers NN193 and NN194, introducing complementary overhangs to pSAM_BT at both ends of the PCR fragment.

The kanamycin-resistance cassette was fused with the linearized pSAM_BT plasmid using Hot Fusion cloning, thereby replacing the erythromycin-resistance cassette with kanamycin resistance. *E. coli* ST18 was transformed with the plasmid pSAM_Kan and further used for the creation of the transposon-mutant library of *P. laumondii* TTO1 through conjugation. Transposon-insertion mutants were selected on LB agar containing kanamycin. All primer sequences are listed in Supplementary Table 15.

Construction of mutant strains. For the deletion of the majority of ArcZ in *P. laumondii* TTO1, a 1,123-bp upstream and a 1,014-bp downstream product were amplified using the primers NN276 and NN277, and NN278 and NN279, respectively. The PCR products were fused using the complementary overhangs introduced by the primers and cloned into the pEB17 plasmid linearized with PstI and BglII. The resulting plasmid was used for transformation of *E. coli* s17-1 λ pir. Conjugation of the plasmid in the *P. laumondii* strains and generation of deletion strains by homologous recombination through counter selection was done as previously described⁴². The deletion mutants were verified by PCR using the primers NN281 and NN282, which yielded a 632-bp fragment for mutants genetically equal to the WT and a 502-bp fragment for the desired deletion mutant. Complementation of the ArcZ deletion was achieved by inserting the full and intact version of ArcZ at the original locus. To do this, a 2,207-bp PCR product including the upstream and downstream region required for homologous recombination and the full-length ArcZ was amplified using the primers NN276 and NN279. The fragment was cloned into pEB17 as described earlier. The verified plasmid construct was used to transform *E. coli* s17-1 λ pir cells. The plasmid was transferred into *P. laumondii* Δ arcZ by conjugation and integrated into the genome of *P. laumondii* Δ arcZ by homologous recombination. The knock-in mutant was generated by a second homologous recombination through counter selection on LB plates containing 6% sucrose. Knock-in mutants were verified by PCR using the primers NN281 and NN282, yielding a 632-bp fragment. The same strategy was used for the construction of the mutant strains in *X. szentirmai*. To generate the promoter-exchange mutants in front of *gxpS*, the plasmid pCEPKMR_ORF00346 was transferred into *X. szentirmai* Δ arcZ and *X. szentirmai* Δ hfq by conjugation and integrated into the genome by homologous recombination.

DNA extraction. Genomic DNA was extracted using the Gentra Puregene yeast/bact. kit (Qiagen) following the manufacturer's instructions. For sequencing of transposon-insertion mutants, genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen).

DNA sequencing and identification of the transposon-insertion site. DNA isolated from the transposon-insertion mutants was sequenced on an Illumina NextSeq platform. DNA libraries were constructed using the Nextera XT DNA preparation kit (Illumina) and whole-genome sequencing was performed using 2 × 150 bp paired-end chemistry. A sequencing depth of >50× was targeted for each sample. Genomes were assembled using SPAdes (v. 3.10.1)⁴³ and annotated using Prokka v. 1.12 (ref. ⁴⁴). The completed genome sequences were analysed and viewed using Geneious v. 6 and 9.1 (<https://www.geneious.com>).

RNA extraction, sequencing and analysis. Pre-cultures of *P. laumondii* TTO1, *X. szentirmai* DSM16338 and their respective ArcZ deletion and knock-in mutants were cultured overnight in LB broth with shaking at 30 °C. The pre-cultures were used the following day to inoculate fresh LB at an OD₆₀₀ of 0.3. The cells were cultured to the mid-exponential phase (the OD values for each experiment can be found in Supplementary Table 3). RNA was extracted using an RNeasy mini kit (Qiagen) following the manufacturer's instructions. To facilitate cell lysis, the cells were pelleted and snap-frozen in liquid nitrogen for 1 min after removing the supernatant. After thawing and resuspending in lysis buffer, the cells were vortexed for 30 s before proceeding with the protocol. The RNA for the sRNA libraries were extracted in duplicate during the mid-exponential phase for *P. laumondii* TTO1 and *X. szentirmai*.

The RNA was sequenced through 150 bp paired-end sequencing by Novogene following rRNA depletion with a RiboZero kit and library preparation following the Illumina protocol for strand-specific libraries. The raw data were trimmed using Trimmomatic⁴⁵ and mapped to the reference genome downloaded from NCBI (NC_005126.1 for *P. laumondii* and NZ_NIBV000000000.1 for *X. szentirmai*) using bowtie2 (v2.3.4.3)⁴⁶. The resulting .sam files were converted to .bam files using samtools (v1.8)⁴⁷ and featureCounts (a part of the subread package)⁴⁸ was used to count reads mapping to annotated genes. The count files were then uploaded to degust (<http://degust.erc.monash.edu/>) and analysed using the voom/limma method of normalization. Only genes with an absolute fold change >2 and false discovery rate <0.01 were considered significantly regulated. Statistical analysis was performed in R (v. 3.6.1) on the degust platform, where the exact code is available to view.

Northern blot analysis. For the northern blot analyses, total RNA was prepared and analysed as described previously⁴⁹. Briefly, the RNA samples were separated on 6% polyacrylamide and 7 M urea gels and transferred to Hybond-XL membranes (GE Healthcare) by electro-blotting. The membranes were hybridized in

Roti-Hybri-Quick buffer (Roth) at 42 °C with gene-specific [³²P] end-labelled DNA oligonucleotides, and washed in three subsequent steps with 5×, 1× and 0.5×SSC (in 0.1% SDS) wash buffer. Signals were visualized on a Typhoon FLA 7000 phosphorimager (FUJIFILM). The oligonucleotides used for the northern blot analyses are listed in Supplementary Table 15.

Compensatory base mutation and GFP fluorescence assay. The plasmids pMH078 and pMH079 were generated using Gibson assembly⁵⁰. For plasmid pMH078, the *arcZ* gene was amplified using *P. laumondii* TTO1 genomic DNA with the oligonucleotides KPO-6147 and KPO-6148, and fused into a pEVS143 vector backbone⁵¹, linearized with KPO-0092 and KPO-1397. To construct the plasmid pMH079, the 5' UTR and the first 20 amino acids of *hexA* were amplified using *P. laumondii* TTO1 genomic DNA with KPO-6145 and KPO-6146, and the pXG10-*gfp* vector⁵² was linearized with KPO-1702 and KPO-1703. The plasmids pMH078 and pMH079 served as templates to insert single point mutations in the *arcZ* gene as well as the *hexA* 5' UTR using site-directed mutagenesis and the oligonucleotide combinations KPO-6156 and KPO-6157, and KPO-6164 and KPO-6165, respectively, yielding the plasmids pMH080 and pMH081.

Target regulation using GFP reporter fusions was analysed as described previously⁵². *E. coli* Top10 cells were cultured overnight in LB medium (37 °C with shaking at 200 r.p.m.). Three independent cultures were used for each strain. The cells were washed in PBS and the GFP fluorescence intensity was determined using a Spark 10M plate reader (TECAN). Samples that did not express fluorescence proteins were used as controls to subtract the background fluorescence.

Cappable-seq analysis. The Cappable-seq was performed as previously described⁵³ by Vertis Biotechnologies. The raw sequences were trimmed using Trimmomatic⁴⁵ and mapped using bowtie2 (ref. ⁴⁶) to NC_005126.1 for *P. laumondii* and NZ_NIBV000000000.1 for *X. szentirmai*. The transcriptional start sites were detected using the built-in TSS detection function read of Xplorer (v2.2.3)⁵⁴ with the following settings: use only single perfect matches, minimum number of read starts = 100, minimum percent coverage increase = 750, detect previously undescribed transcripts, minimum transcript extension = 40, maximum distance to feature of leaderless transcripts = 5 and associate neighbouring TSS within 3 bp.

RIP-seq analysis. Overnight cultures of *P. laumondii* TTO1 (WT and Hfq^{3xFLAG}) were inoculated into fresh LB medium in duplicate and cultured at 30 °C with shaking at 200 r.p.m. The bacteria were harvested by centrifugation at 4,000 r.p.m. for 15 min at 4 °C when the cells reached OD₆₀₀ = 0.5 and OD₆₀₀ = 5.0. The cell pellets were resuspended in 1 ml lysis buffer (20 mM Tris pH 8.0, 150 mM KCl, 1 mM MgCl₂ and 1 mM dithiothreitol) and pelleted again by centrifugation (5 min, 11,200g, 4 °C). The supernatants were discarded and the pellets were snap-frozen in liquid nitrogen. After thawing on ice, the cells were resuspended in 800 µl lysis buffer and transferred into tubes containing 300 µl glass beads to break the cells using a Bead Ruptor (2 × 150 s, with a 2 min break on ice in between). After a short centrifugation (15,000g, 4 °C), the lysates were transferred into fresh pre-cooled tubes and centrifuged for 30 min at 15,200g at 4 °C. The cleared lysates were transferred into fresh tubes and incubated with 35 µl FLAG-antibody (monoclonal anti-FLAG M2; Sigma, F1804) with rotation for 45 min at 4 °C, followed by the addition of 75 µl Protein G Sepharose (Sigma, P3296) and rotation for 45 min at 4 °C again. After five wash steps with lysis buffer (by inverting the tube gently and centrifuging for 4 min at 4 °C), the samples were subjected to RNA and protein separation using phenol:chloroform:isoamylalcohol (25:24:1, pH 4.5; Roth) extraction. The upper phase (approximately 500 µl) was transferred into a fresh tube and precipitated overnight at -20 °C with 1.5 ml EtOH:Na(acetate) (30:1) and 1.5 µl GlycoBlue (Ambion, AM9516). After centrifugation at 11,200 r.p.m. for 30 min at 4 °C, the RNA pellets were washed with 500 µl 70% EtOH, dried and resuspended in 15.5 µl nuclease-free H₂O. The RNA was treated with 2 µl DNase I, 0.5 µl RNase inhibitor and 2 µl 10×DNase buffer at 37 °C for 30 min. The samples were then supplemented with 100 µl H₂O and again subjected to a phenol:chloroform:isoamylalcohol extraction. The upper phase (approximately 120 µl) was transferred to a fresh tube with the addition of 2.5–3 volumes (about 350 µl) of EtOH:Na(acetate) (30:1) and stored at -20 °C overnight for RNA precipitation. The RNA pellets were harvested via centrifugation at 13,000 r.p.m. for 30 min at 4 °C, and washed with 500 µl of 70% EtOH, dried and resuspended in nuclease-free H₂O. Complementary DNA libraries were prepared using a NEBNext small RNA library prep set for Illumina (NEB, E7300S) according to the manufacturer's instructions and sequenced on a HiSeq 1500 system in single-read mode with a read length of 100 nucleotides.

For RIP-seq analysis, the enriched-control sample pairs were normalized to the number of raw reads present after trimming. Depth counts of all samples were obtained using samtools (v1.8)⁴⁷. Only nucleotide positions with a depth of at least 50 reads in the enriched samples were taken for further analysis. The corresponding depth in the unenriched samples was matched for each nucleotide. A region was considered to be enriched if the enrichment factor was at least three and the corresponding 'enriched' nucleotide was present in both sample pairs. Finally, we considered a region to be enriched if more than five consecutive nucleotides were identified as enriched.

ArcZ-binding prediction. ArcZ from *E. coli* was used to define the boundaries of ArcZ in *Xenorhabdus* and *Photorhabdus*. We then took our annotated ArcZ sequence together with several ArcZ homologues from other Enterobacteriaceae (listed in Supplementary Table 9) and used the online CopraRNA tool²³, a part of the Freiburg RNA tools suite²⁶, with default parameters.

Metabolite extraction and HPLC–MS/MS analysis. Fresh LB medium (10 ml) was inoculated with an overnight culture to an OD₆₀₀ of 0.1. After 72 h of cultivation at 30 °C with shaking, 1 ml of the culture was removed from the culture, centrifuged for 20 min at 13,300 r.p.m. and the supernatant was directly subjected for analysis by HPLC–MS/MS using a Dionex Ultimate 3000 system with a Bruker AmaZon X mass spectrometer. The peak areas of the compounds were quantified using TargetAnalysis 1.3 (Bruker). All of the analysed compounds are listed in Supplementary Table 16.

Proteome analysis. The details of the proteomics procedure were previously published⁵⁶. In short, to extract proteins from *P. laumondii* frozen cell pellets, 300 µl lysis buffer (0.5% Na-desoxycholate in 100 mM NH₄HCO₃) was added to the cell pellet and incubated at 95 °C for 10 min. The protein concentration in the supernatant was determined using a BCA protein assay kit (Thermo Fisher, 23252). Reduction and alkylation was performed at 95 °C using 5 mM TCEP and 10 mM chloroacetamide for 15 min. The protein (50 µg) was transferred to fresh reaction tubes and protein digestion was carried out overnight at 30 °C with 1 µg trypsin (Promega). After the digestion, the peptides were desalted using CHROMABOND spin columns (Macherey–Nagel) that were conditioned with 500 µl acetonitrile and equilibrated with 500 µl and 150 µl 0.1% trifluoroacetic acid (TFA). After loading, the peptides were washed with 500 µl of 0.1% TFA in acetonitrile:H₂O (5:95) and eluted with 400 µl of 0.1% TFA in acetonitrile:H₂O (50:50). The peptides were concentrated and dried under vacuum at 50 °C and dissolved in 100 µl of 0.1% TFA by 25 s of sonication and incubation at 22 °C with shaking at 1,200 r.p.m. for 5 min. Peptides (1 µg) were analysed using HPLC–MS/MS.

The HPLC–MS/MS analysis including label-free quantification was carried out as previously described⁵⁶, with minor modifications.

HPLC–MS/MS analysis of protein digests was performed on a Q-Exactive Plus mass spectrometer connected to an electrospray ion source (Thermo Fisher Scientific). Peptide separation was carried out using an Ultimate 3000 nanoLC-system (Thermo Fisher Scientific) equipped with C18 resin column packed in-house (Magic C18 AQ 2.4 µm; Dr. Maisch). The peptides were first loaded onto a C18 pre-column (pre-concentration set-up) and then eluted in backflush mode with a gradient from 98% solvent A (0.15% formic acid) and 2% solvent B (99.85% acetonitrile and 0.15% formic acid) to 35% solvent B over 30 min. Label-free quantification was done using Progenesis QI software (Nonlinear Dynamics, v2.0) and the MS/MS search was performed in MASCOT (v2.5, Matrix Science) against the UniProt *P. laumondii* protein database. The following search parameters were used: full tryptic search with two missed cleavage sites, 10 ppm MS1 and 0.02 Da fragment ion tolerance. Carbamidomethylation (C) as fixed, and oxidation (M) and deamidation (N,Q) as variable modifications. The progenesis outputs were further processed using SafeQuant⁵⁷.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All .mzXML files from the HPLC–MS/MS runs are available at MassIVE (<https://massive.ucsd.edu>) under the ID MSV000084163. Raw sequence data are available at the European nucleotide archive (<https://www.ebi.ac.uk/ena/>) under project accession numbers PRJEB33827 and PRJEB24159. The proteomic data can be accessed at PRIDE (<https://www.ebi.ac.uk/pride/>) with the project accession number PXD019095. Source data are provided with this paper.

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Author contributions

N.N., N.J.T., M.H., X.C., A.L.L., T.G. and S.J.P. performed the experiments, except sequencing of the transposon-insertion mutants, which was performed by S.J.P. and T.P.S. N.N., N.J.T., M.H., K.P. and H.B.B. designed the study, discussed the results and commented on the manuscript. N.N., N.J.T. and M.H. analysed and interpreted the data. N.N., N.J.T. and M.H. wrote the manuscript. All of the authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | Northern blot analysis of various sRNAs in *P. laumondii*. Expression of various sRNAs in *P. laumondii* at different time points. RNA samples of *P. laumondii* WT and Δhfq strains were taken at three different OD₆₀₀ values (0.5, 2 and 4) and after 24 h of growth. The RNA was loaded on northern blots and probed for the indicated sRNAs. Probing for 5S rRNA served as loading control.

Extended Data Fig. 2 | Northern blot analysis of various sRNAs in *X. szentirmaii*. Expression of various sRNAs in *X. szentirmaii* at different time points. RNA samples of *X. szentirmaii* WT and Δhfq strains were taken at three different OD₆₀₀ values (0.5, 2 and 4) and after 24 h of growth. The RNA was loaded on northern blots and probed for the indicated sRNAs. Probing for 5S rRNA served as loading control.

a

b

Extended Data Fig. 3 | See next page for caption.

Extended Data Fig. 3 | Phenotype and specialized metabolite profile of transposon-insertion mutants of *P. laumondii*. Phenotype of transposon-insertion mutants of *P. laumondii*. **a.** Differences in pigmentation of transposon-insertion mutant liquid cultures compared to WT. Depicted are eleven transposon-insertion mutants and a WT culture after 3 d of cultivation at 30 °C with shaking. **b.** SM profiles of the transposon-insertion mutants. Relative SM production was quantified from duplicates using TargetAnalysis (Bruker) and compared to the WT of *P. laumondii* after 72 h cultivation at 30 °C with shaking. Mutant 3 was analysed further and the transposon insertion was identified in the *arcZ* gene.

Extended Data Fig. 4 | Nematode bioassay. Infective juvenile development to hermaphrodites with strains of *P. laumondii* and *X. szentirmai*. Data are presented as the mean $\pm$ s.e.m. Dots represent biologically independent replicates ($n=10$). Asterisks indicate statistical significance (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.00005$) of relative recovery compared to WT recovery levels. Statistical significances were calculated using a two-sided unpaired t-test. Exact p values (left to right, respectively) for *P. laumondii* TTO1 correspond to $p = <0.0001$, 0.0006 and for *X. szentirmai* to $P = 0.56$, 0.0094.

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Data collection	During HPLC/MS analysis, Chromeleon Chromatography Data System (CDS) was used to collect HPLC data and HyStar (v 3.2) for MS data.
Data analysis	Genomes were assembled with SPAdes (v3.10.1) and annotated with Prokka (v1.12). Completed genome sequences were analysed and viewed in Geneious v9.1 and v6. Raw sequencing data was trimmed using Trimmomatic (v0.39), mapped using bowtie (v2.3.4.3) converted to bam using samtools (1.8) and counted using featureCounts (a part of the subread package (v1.6.5)). Statistical analysis was performed in R (v 3.6.1) on the degust platform (http://degust.erc.monash.edu/), where exact code is available to view. Transcriptional start sites were detected using readXplorer (V2.2.3). CopraRNA (v2.1.2) was used to predict ArcZ binding sites. Chemical compound analysis was performed using The compounds peak areas were quantified using TargetAnalysis (v1.3). Label-free quantification was performed using the Progenesis Q1 software (Nonlinear Dynamics, v2.0). Progenesis output was processed with SafeQuant (v2.3.1).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	For sequencing experiments, duplicates were used. Based on our previous experience this has been sufficient to determine significant differences using strict false-discovery cutoffs (https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/1462-2920.13502 ; https://www.nature.com/articles/s41589-019-0246-1#Sec10). Chemical compound analyses were performed in triplicate to confirm observations. Based on our experience, the relative production of natural products throughout replicates does not fluctuate very much, as also indicated by the standard deviations shown in this paper and previous work (https://pubmed.ncbi.nlm.nih.gov/28261170/). Nematode bioassays were performed in ten replicates. Sample sizes for nematode bioassays were chosen according to previously published work (https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/1462-2920.13502).
Data exclusions	No data were excluded from analysis.
Replication	All sequencing experiments were performed in duplicate followed by normalization and statistical analyses. We confirmed several outcomes defined by predictive tools by using molecular methods (ie. specific DNA modifications). Nematode bioassays and compound analyses were performed independently, in triplicate to confirm observations.
Randomization	All experiments were designed to elucidate a specific functional mechanism. Randomization was not relevant to this study because there was no group allocation involved.
Blinding	Blinding was not relevant to this study. The majority of sequencing experiments were RNA based and the results therefore are definitive. No modification of any data was performed and all results are reported as direct outputs of individual analyses.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Monoclonal ANTI-FLAG M2 antibody, Sigma, #F1804, Lot# SLBT7654, produced in mouse
Validation	commercially available antibody; purified IgG1 monoclonal antibody that binds to fusion proteins containing a FLAG peptide sequence