1 **Temperature restriction in entomopathogenic bacteria**

2

3 **Alexia Hapeshi¹✉, Joseph R.J. Healey¹, Geraldine Mulley² and Nicholas R.**
 4 **Waterfield¹***

5

6 ¹Microbiology and Infection Unit, Warwick Medical School,

7 The University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK.

8 ²School of Biological Sciences, University of Reading, Whiteknights, Reading RG6

9 6AJ, UK.

10 *** Correspondence:**

11 Corresponding Author

12 n.r.waterfield@warwick.ac.uk

13

14 ✉Present address: Chemistry Department, University of Warwick, Gibbet Hill Road,
 15 Coventry, CV4 7AL, UK.

16

17 **Keywords: temperature, adaptation, enterobacteria, *Photorhabdus*, symbionts,**
 18 **pathogens**

19

20 **Abstract**

21

22 Temperature plays an important role in bacteria-host interactions and can be a
 23 determining factor for host switching. In this study we sought to investigate the reasons
 24 behind growth temperature restriction in the entomopathogenic enterobacterium
 25 *Photorhabdus*. *Photorhabdus* has a complex dual symbiotic and pathogenic life cycle.
 26 The genus consists of 19 species but only one subgroup, previously all classed together
 27 as *P. asymbiotica*, have been shown to cause human disease. These clinical isolates

necessarily need to be able to grow at 37 °C, whilst the remaining species are largely restricted to growth temperatures below 34 °C and are therefore unable to infect mammalian hosts. Here, we have isolated spontaneous mutant lines of *P. laumondii* DJC that were able to grow up to 36 °C- 37 °C. Following whole genome sequencing of 29 of these mutants we identified a single gene, encoding a protein with a RecG-like helicase domain, that for the majority of isolates contained single nucleotide polymorphisms. Importantly, provision of the wild-type allele of this gene in *trans* restored the temperature restriction, confirming the mutations are recessive, and the dominant effect of the protein product of this gene. The gene appears to be part of a short three cistron operon, which we have termed the Temperature Restricting Locus (TRL). Transcription reporter strains revealed that this operon is induced upon the switch from 30 °C to 37 °C, leading to replication arrest of the bacteria. TRL is absent from all of the human pathogenic species so far examined, although its presence is not uniform in different strains of the *P. luminescens* subgroup. In a wider context, the presence of this gene is not limited to *Photorhabdus*, being found in phylogenetically diverse proteobacteria. We therefore suggest that this system may play a more fundamental role in temperature restriction in diverse species, relating to as yet cryptic aspects of their ecological niches and life cycle requirements.

1 Introduction

Photorhabdus is a Gram-negative bacterium that forms a symbiotic relationship with *Heterorhabditis* nematodes. The *Heterorhabditis-Photorhabdus* complex is entomopathogenic with the nematodes infecting insects and releasing the bacteria by regurgitation (Ciche and Ensign 2003). *Photorhabdus* then sets up a lethal septicaemia, producing an array of toxins and degradative enzymes that combat the immune system and kill the insect. They also elaborate a potent cocktail of antimicrobial compounds to eliminate competition by other microbes in the carcass. The bacteria reproduce using nutrients derived from the insect tissues, reaching very high numbers. The nematodes feed on this bacterial biomass, going through several rounds of hermaphroditic reproduction, before eventually re-establishing the symbiosis with the bacteria (reviewed in (Waterfield, Ciche, and Clarke 2009)). The

Photorhabdus genus, which belongs to the class of γ -proteobacteria, was thought to consist of three species *P. asymbiotica*, *P. luminescens* and *P. temperata*. Recently, however, it has been proposed that several of the subspecies be elevated to species level resulting in a total of 19 species (Machado et al. 2018). Nevertheless, for the purposes of this study we will refer to these as belonging to the *P. asymbiotica*, *P. luminescens* and *P. temperata* subgroups. Thus, so far, only species belonging to the *P. asymbiotica* subgroup (that can grow up to 37 °C or above) have been identified as the causative agent of human infections (Gerrard and Stevens 2017). The only exception to this was a strain of *P. luminescens*, identified using 16S sequencing, which was associated with cutaneous lesions and bacteremia in a hypothermic infant with a body temperature upon admission of 33.6 °C (Dutta et al. 2018). No other members of the subgroups *P. luminescens* and *P. temperata*, the majority of which are largely restricted to growth temperatures below 37 °C (Fischer-Le Saux et al. 1999; Tailliez et al. 2010; Machado et al. 2018), have been reported to cause human disease. The exact reasons behind this discrepancy are unknown.

The establishment and maintenance of symbiosis is also a very important aspect of the *Photorhabdus* life cycle. There is considerable partner preference in the association between *Photorhabdus* bacteria and the *Heterorhabditis* nematodes, although this is not strictly species-specific (Maher et al. 2017; Kazimierczak et al. 2017). Nonetheless, there is evidence for the co-evolution of the two partners (Maneesakorn et al. 2011). It may therefore be of relevance, that the optimum temperature for the nematodes is also relatively low. For example, in our hands the *P. laumondii* DJC *Heterorhabditid* nematode partner dies in culture above 34 °C (unpublished data).

P. laumondii sbsp. *laumondii* DJC is a laboratory strain of *Photorhabdus* which is normally unable to grow at temperatures above 35 °C on solid LB media. In this study we have isolated mutants of *P. laumondii* DJC with abilities to grow between 36-37 °C.

2 Results

2.1 Isolation and characterisation of temperature tolerant *P. laumondii* DJC mutants

Clones of *P. laumondii* DJC were isolated with the ability to grow on LB agar at 36 °C. This was following plating of a saturated overnight culture and incubation of the plates at the higher temperature. The process was repeated two more times and colonies were isolated from all three experiments originating from four independent lineages in total. Isolates were re-streaked 5 times at 36 °C to select for stably growing clones. Overall, the average frequency with which stable clones were isolated was $2.6 \pm 2.5 \times 10^{-6}$. Thirty of those were then analysed by whole genome sequencing to identify any mutations that may give rise to the phenotype. Sequencing was performed using the MiSeq platform and good quality reads were obtained for 29 of the 30 clones. The WT strain in the lab was also sequenced to account for variations between isolates in different laboratories. Reads from the temperature tolerant clones were then mapped against the reference to identify single nucleotide polymorphisms (SNPs).

Of the 29 isolates successfully sequenced, 19 had a SNP in an uncharacterised gene *PluDJC_RS01875* (Figure 1A and Table 1), and an additional isolate (S15) had an IS30-family transposase insertion near the 3' end (chromosomal position 393017). This gene appears in the automated annotation as “*recG*”, however it should be noted that a full-length orthologue of the bona fide *recG* is encoded elsewhere in the genome. RecG is a 693 amino acid protein whose homologue in other bacteria has been well studied and is known to be important for DNA repair and recombination (Ishioka, Iwasaki, and Shinagawa 1997; Rudolph et al. 2010). As *PluDJC_RS01875* is involved in the temperature restriction phenotype, and to distinguish it from the *recG* misnomer, we subsequently named it *trlG* (temperature restriction locus G).

Repetition of these experiments aimed at isolating temperature tolerant clones by a collaborator in an independent laboratory (Dr G. Mulley), gave rise to two additional

tolerant isolates, named JA and JB. These were specifically tested for the presence of mutations in *trlG* by sequencing of a PCR amplicon. Clone JA was found to contain a SNP, while in clone JB an IS66 transposable element had inserted into the 3' end of *trlG* (chromosomal position 393014). Homology and structural models of the TrlG protein suggest it comprises two distinct functional domains connected by a flexible loop region. Most of the mutant SNPs were located in either of the two domains. Additionally, three mutants exhibited premature stop codon mutations in the linker region (Figure 1B). Based on a 3D structure prediction of the protein, we can observe that four of the SNPs would result in substitutions in alpha helices potentially disrupting the structure (Figure 1C). The remaining isolates that did not have a mutation in *trlG* were actually unable to resume growth at 36 °C after being frozen (Figure 2A and Supplementary Figure 1). It should be noted that the tolerant isolates are less pigmented and produce less bioluminescence at the higher temperature (Figure 2A and Supplementary Figure 2). We then investigated the hypothesis that the operon might affect growth or survival at colder temperatures by incubating the bacteria at lower temperatures before shifting back to 28 °C. However, the mutants in this case behaved similarly to the WT bacteria (data not shown).

2.2 The *trlG* gene is part of a small putative three gene operon.

Examination of the genomic context of *trlG* revealed it is part of a small operon with *PluDJC_RS01880* and *PluDJC_RS01885*, encoded between the DNA polymerase gene, *polA*, and *dsbA* (Figure 1A). *PluDJC_RS01880* encodes a protein annotated as a histidinol phosphatase (WP_011144772.1). The product of *PluDJC_RS01885* (WP_011144773.1) on the other hand is a small 79 amino acid protein annotated as a hypothetical protein but homologues in other organisms are annotated as transcription factors.

To further confirm the role of *trlG* in temperature restriction we constructed an unmarked deletion mutant of the entire TRL operon, Δtrl . This mutant was also able to grow on both solid and liquid media at 36 °C (Figure 2B). Importantly, plasmid trans-complementation with the whole operon restored the temperature restricted

phenotype in the SNP mutants (Figure 3). Note, we used the whole operon rather than just *trlG* alone to ensure the stoichiometry of the three gene members was preserved.

Finally, since virulence of the bacteria to insects is a crucial aspect of their life cycle, we determined if mutation of *trlG* would have any effect on virulence against *Galleria mellonella* larvae. The experiment was performed at room temperature (~20 °C), using a representative SNP mutant as well as the Δtrl deletion strain, compared to WT and the Rif^r isogenic parent strains respectively. Interestingly, we observed that the SNP mutant killed the *Galleria* larvae much faster than the WT (Figure 4). This was also the case for the Δtrl , albeit the effect was less pronounced. We also performed this virulence assay at 36 °C. As expected the WT strain was not able to kill the *Galleria*, although surprisingly, even though they could grow at this temperature *in vitro*, the mutant strains also failed to establish an infection.

2.3 Transcription of the TRL operon is induced by temperature up-shift

To determine whether the TRL operon is specifically expressed in response to exposure to the higher temperature we created reporter constructs whereby we cloned regions upstream of the operon upstream of a promoterless GFP gene. An *in silico* search for promoters using BPROM (Solovyev and Salamov 2011) showed the presence of two putative promoters upstream of the first gene in the TRL operon (*PluDJC_RS01880*, designated here as *trlR*). Interestingly, one of these promoters seems to be upstream of the predicted, oppositely encoded *polA* promoter. Analysis of apparent transcription start sites in our unpublished RNAseq analysis of *P. laumondii* supports the location of these predicted promoters. Additionally, a putative promoter is predicted which is encoded inside the *trlR* open reading frame. With TrlR itself showing homology to a transcriptional regulator we hypothesised that this might be a legitimate promoter driving expression of *trlF* and *trlG*. We therefore decided to create multiple constructs to ensure that we capture the transcriptional elements that control expression of genes in the TRL operon (Figure 5A). These constructs were separately introduced back into a rifampicin resistant isolate of DJC and the effect of

temperature up-shift tested. These experiments confirmed that transcription of the operon is increased upon temperature up-shift (Figure 5B and 5C). It should be noted that the growth of *P. laumondii* DJC following temperature up-shift does not immediately cease, but rather continues to increase briefly before reaching a plateau (Supplementary figure 3A). Moreover, at the end of the 24 h period and following a ~20 h incubation at the higher temperature, a large proportion of cells remain viable as shown by live/dead staining (Supplementary figure 3B). Importantly, the expression of these reporter constructs when the bacteria are grown at 28 °C was also monitored and we observed that the operon is also expressed at the permissive growth temperature but expression shows a gradual increase as the bacteria go through late exponential and stationary phase (Figure 5B and 5C).

2.4 Presence of the TRL in the *Photorhabdus* genus

Since mutations in *trlG* appear to allow *P. laumondii* to survive in higher temperatures we were interested in investigating the presence of the gene among other *Photorhabdus* genomes. A *trlG* homologue was found in 21 out of the 46 *Photorhabdus* genomes in the RefSeq genome database (Figure 6 and Supplementary Figure 4). However, no *trlG* homologues were present in any bacteria belonging to the *P. asymbiotica* or *P. temperata* subgroups. Within the group of bacteria previously classed as *P. luminescens* the presence of the gene is not uniform. Those isolates that possess the gene actually encode for the full operon. Even though the majority of those genomes are in the draft sequencing stage, we investigated the location of the operon in the genome and observed that it appears to be conserved in the region between *dsbA* and *polA*. Importantly, in the published genome sequence of the type strain *P. laumondii* (Duchaud et al. 2003) an insertion sequence (IS) element is found upstream of the *polA* gene suggesting the operon may have been horizontally acquired. However, we note that this IS element is absent in our WT *P. laumondii* DJC strain – which is a laboratory selected derivative of the original TT01 isolate (confirmed by PCR). Interestingly, Alien Hunter analysis (Vernikos and Parkhill 2006) shows no evidence of horizontal gene transfer of this three gene TRL locus, suggesting it is ancestral in this sub-lineage of *Photorhabdus*. In addition to *P.*

laumondii TT01, only *P. laumondii* strain DSPV002N possesses an IS630-like element ISPlu10 family transposase directly upstream of the operon. Four other isolates, *P. laumondii* HP88, *Photorhabdus* sp. S7-51, *Photorhabdus* sp. S15-56 and *Photorhabdus* sp. S14-60, appear to possess a gene encoding for a 61 amino acid hypothetical protein (WP_133148736.1), upstream of the operon but in the opposite strand.

2.5 The TRL is conserved amongst bacterial genera

The TRL is of relevance to bacteria beyond the *Photorhabdus* genus. Blastp analysis against the NCBI reference protein database showed that TrlG is prevalent in proteobacteria (Figure 7). The closest homologue outside of *Photorhabdus* is found in *Vibrio parahaemolyticus* followed by proteins encoded by *Pseudospirillum japonicum* and *Nitrincola nitratireducens*, which like *Photorhabdus* belong to the class of γ -proteobacteria. TrlG was, however, absent from the closely related *Xenorhabdus*, also a nematode endosymbiont. While the TrlG proteins of *Photorhabdus* and *Glaeserella* are all clustered together, for other bacteria this does not seem to be the case, suggesting individual acquisition events. Within the *Vibrio* genus, the gene is found in specific isolates of different species, such as *V. cholerae*, *V. anguillarum* and *V. atlanticus*. As an example, out of the 1218 *V. cholerae* genomes found in the RefSeq database, only 12 were found to encode for a homologue of TrlG (with over 40 % sequence identity). Of note is the presence of TrlG in various *Pseudomonas* isolates. Some are classified as strains of the plant pathogens *Pseudomonas syringiae* or *Pseudomonas amygdali*, however the gene is also present in some isolates of *P. aeruginosa*.

Analysis of conservation of the entire operon using MultiGeneBlast (Medema, Takano, and Breitling 2013) against a Genbank database consisting of complete bacterial genomes showed that outside of the *Photorhabdus* genus, all three genes, *pluDJC_RS01875 - RS01885* are only conserved syntenically in strains of *Vibrio anguillarum* and on plasmid pYR3 of *Yersinia ruckeri* strain CSF007-82 (locus tags

CSF007_p0360, CSF007_p0365, CSF007_p0345, Figure 8). However, unlike the TRL of *P. laumondii* where the three genes are located in succession, in pYR3 there are two small genes annotated as encoding Type I restriction-modification system, DNA-methyltransferase subunit M in between CSF007_p0345, the gene encoding the putative transcriptional regulator, and CSF007_p0360. *Yersinia ruckeri* is the causative agent of enteric red mouth disease in salmonid fish species (Kumar et al. 2015) and its optimum growth temperature is also 28 °C, while its optimum temperature for infectivity is 18 °C. Similarly, *V. anguillarum* is also a fish pathogen with an optimum growth temperature of 25 °C whilst the optimum temperature for vibriosis disease is 15 °C (Lages, Balado, and Lemos 2019). It appears that a lower temperature optimum for virulence is common in fish pathogens and virulence genes are found to be up-regulated at these lower temperatures (Guijarro et al. 2015; Mendez et al. 2018; Lages, Balado, and Lemos 2019).

3 Discussion

Members of the genus *Photothabdus* dedicate a significant proportion of their genome to the synthesis of virulence factors and resource defence. Indeed, when the first *Photothabdus* genome sequence was completed it was noted that there were more predicted toxin genes than in any other bacteria that had been sequenced at the time (Duchaud et al. 2003). In addition, many other genera belonging to the γ -proteobacteria contain multiple examples of mammalian pathogens, both obligate and opportunistic. With these observations in mind it is perhaps unusual that only very few members of the genus *Photothabdus* are able to cause infection in humans (Gerrard and Stevens 2017). Our previous analysis of the adaptations relevant to human pathogenicity in a representative *P. asymbiotica* strain suggested that no specific mammalian active toxins are necessary to facilitate human infection. Rather it seems that substantive changes in metabolic activity, when cultured at 37 °C, are primarily responsible for their expanded host range (Mulley et al. 2015); a phenomenon termed nutritional virulence (Abu Kwaik and Bumann 2013).

An obvious limitation for mammalian infection in many members of the genus is their inability to grow above ~35 °C, which again is unusual for members of the γ -proteobacteria. Typically, maximum growth temperatures of bacteria are determined by multiple factors that affect bacterial physiology, such as membrane and lipid fluidity, chaperone activities and other enzyme/protein functions. It is therefore possible that for any one species there are multiple functions that fail simultaneously at the non-permissive temperature. However, the ability of *P. asymbiotica* isolates to grow readily at temperatures of 37 °C (and above) and the overall high level of conservation in core genome sequence across the genus indicates that there is likely no general physiological reason for temperature restriction in members of the genus.

Our identification of the TRL genes offers up an alternative explanation to this temperature restriction phenomenon, at least in certain lineages of *P. laumondii*. The specific induction of the TRL gene transcription upon temperature up-shift, leading to growth arrest, appears *prima facie* to represent a specific adaptive mechanism. We do not yet know if induction of TRL transcription at higher temperatures is mediated by the TrlR protein alone or if other regulatory pathways are involved. While it remains unclear as to what advantage this temperature dependent growth arrest imparts upon these strains, we may speculate. For example, we note that the nematode symbiont cannot survive above ~34 °C *in vitro*, although to our knowledge nematode survival at different temperatures *in vivo* has not been investigated. Therefore, a TRL mediated mechanism to suppress continued exponential growth at increasing temperatures, may represent a mechanism to synchronise life cycles of the bacteria and nematode in fluctuating environmental conditions. It is perhaps no coincidence that the TRL operon is encoded next to the *polA* gene and that the most 5' TRL promoter overlaps on the opposing strand with that of the *polA* gene. Thus, activation of TRL transcription might directly suppress *polA* transcription by steric hindrance. It is formally possible that the tight linkage between *polA* and TRL reflects a functional linkage in the gene activities (ie, replication and temperature). It is interesting in this respect that even at 28 °C, we see a gradual increase in TRL transcription as the culture moves toward stationary phase and the eventual cessation of replication.

Interestingly, while ablation of the whole TRL operon did not strongly affect virulence in the *Galleria* infection model at 28 °C, the S1 SNP mutant actually became significantly more virulent at this temperature. This suggests a functional interplay between *trlG*, *trlF* and *trlR*, as only the *trlG* gene is mutated in S1. Therefore, the activity of *trlF* and/or *trlR* in the absence of the balancing effect of *trlG* seems to enhance virulence activity. Importantly, when the host temperature was increased to 36 °C, the infection failed of both the S1 SNP and Δtrl strains, despite their ability to grow *in vitro*. This is consistent with the reported failure of *P. asymbiotica* to establish a lethal infection in the *Manduca sexta* model when incubated at 37 °C (Mulley et al. 2015). In a natural context 37 °C is not a problem for *P. asymbiotica* ATCC43949 as it would be in a mammalian host, for which it is adapted. Thus, despite the alleviation of growth restriction, there are clearly other temperature dependent changes in gene regulation occurring, as evidenced by the down regulation of luciferase activity and pigment production in these mutants. This effect may be common to both *P. asymbiotica* and the mutant *P. laumondii* lineages and regulated through a separate unknown mechanism. In addition, the loss of pigmentation and reduction in bioluminescence of the mutant colonies at 36 °C is reminiscent of the secondary phenotypic variants of *Photorhabdus* (Boemare and Akhurst 1988) as well as Δmdh and $\Delta fumC$ mutants deficient in enzymes of the TCA cycle (Lango and Clarke 2010) and a $\Delta relAspoT$ strain, deficient in (p)pGpp synthesis (Bager et al. 2016). The aforementioned mutants retain virulence in insect models but are no longer symbiotically competent. Absence of pigmentation and bioluminescence is also a characteristic of the M-phase variants of *P. luminescens*, which are responsible for transmission of the bacteria to infective juvenile nematodes and establishment of symbiosis, however those variants are avirulent against insects (Somvanshi et al. 2012).

The majority of random mutations selected from the population were non-synonymous amino acid substitutions in TrlG rather than premature stop codons or frame shifts. It is possible that this simply reflects the lower probability of obtaining a stop codon by random chance as complete deletion of the operon suggested the presence of an altered version of TrlG was not in some way advantageous. More

importantly, no mutations were seen in the much larger and tightly linked *trlF* gene. This suggests that either (i) TrlF does not play a role in the temperature restriction phenotype, or (ii) that if it does, its function can be fully compensated by dominant activity of an intact TrlG protein. The small size of the *trlR* regulator gene means there was a much lower probability of isolating mutants in this. As we could delete the whole operon, it indicates that mutations in *trlF* alone are not overtly deleterious if they should occur in isolation.

As the lifestyles of most *Photorhabdus* strains are generally similar, it is interesting that the TRL operon appears restricted yet ancestral to a specific species lineage of *Photorhabdus*. As members of this lineage are geographically dispersed (Machado et al. 2018; Mathur et al. 2018), factors such as biogeography, insect prey type or the nature of competing organisms are unlikely to be the reason for maintaining TRL. We therefore speculate that selection for TRL may be a result of specific requirements of the host bacterial genomes, or perhaps in restrictions imposed by the nematode host lineage. For example; as the *P. luminescens* TT01 genome is heavily infested by IS-elements, we could hypothesise that TRL represents a mechanism to protect against rampant IS-element activity. If a sustained temperature increase was “perceived” as stress to the bacteria (or more importantly the IS-elements), it may lead to undesirable activation of a large number of IS-elements. Therefore, stopping genome replication would likely protect against extensive transposition damage.

An alternative idea is that these strains rely on the activities of a pathway or global regulator which is highly sensitive to temperature. For example, it has been shown that secondary metabolite production is reliant upon the activity of the Hfq protein (Tobias et al. 2017) which, in other Gram-negative bacteria studied, mediates interactions of regulatory ncRNAs with their target mRNA molecules (A. Zhang et al. 1998; Møller et al. 2002; Lease and Woodson 2004). Folding and interactions of many ncRNAs are often temperature sensitive. The lack of pigment and bioluminescence in the mutant strains at the higher temperature suggests that this regulation is indeed being perturbed. If all secondary metabolism necessary for virulence and symbiosis ceases at the higher temperature it would make sense for the bacterium to inhibit further replication until normal regulation is re-established.

373

374 Finally, the identification of TRL-like operons in members of other genera suggests
375 this represents a widespread general mechanism, regulating the life-cycles of
376 phylogenetically diverse bacteria. The association of TRL operons with pathogens of
377 poikilothermic hosts (e.g. fish) tempts speculation there is an as yet unknown
378 advantage to this mechanism in such niches.

379

380 **4 Materials and Methods**

381

382 **4.1 Strains and growth conditions**

383 The strains used in this study were the rifampicin sensitive parent strain of *P.*
384 *laumondii* subsp. *laumondii* DJC (WT) (Zamora-Lagos et al. 2018), and a rifampicin
385 resistant derivative of this strain, *P. luminescens* spbsp. *laumondii* DJC (Rif^R) (Jones
386 et al. 2010). Bacteria were grown in Lysogeny broth (LB) at 28 °C unless otherwise
387 stated. For growth on solid media, LB was supplemented with 1.5 % agar and 0.1 %
388 sodium pyruvate and plates were incubated in the dark. When necessary, kanamycin
389 was added to the media at a concentration of 25 µg/ml. To assess growth on solid
390 media, overnight cultures of *Photorhabdus* were diluted to an OD₆₀₀ of 0.05 and 5 µl
391 were spotted on solid media. They were then incubated at the appropriate temperature
392 for 48 h. To assess growth in liquid, the diluted cultures were incubated at the
393 appropriate temperature for 24 h, shaking at 180 rpm.

394

395 **4.2 Isolation of the temperature tolerant clones**

396 Saturated overnight cultures of *P. laumondii* DJC grown at 28 °C in LB medium were
397 plated on LB agar (LBA) and incubated at 36 °C. After approximately 48 h colonies
398 were observed on the plates. These were re-streaked 4 times on LBA at 36 °C and
399 stably grown clones were stored at -80 °C in 32 % v/v glycerol.

400

4.3 Whole-genome sequencing and bioinformatics analysis

DNA was isolated from 1 ml of overnight cultures of *P. laumondii* DJC using the Qiagen Blood and Tissue kit. The Nextera XT kit (Illumina) was used for library preparation and sequencing was performed using a MiSeq v2 cartridge on a MiSeq sequencer (Illumina). Raw data has been deposited in the NCBI database under BioProject ID PRJNA596169, while raw sequencing reads for the WT isolate in our lab can be found in the short read archive using accession number SRR8307122. Fastq reads of both the WT and the tolerant clones were mapped against the NCBI reference sequence for *P. luminescence* subsp *laumondii* DJC NZ_CP024900.1 (Langmead and Salzberg 2012) in very sensitive mode. SAMtools v0.1.18 (Li et al. 2009) was used to obtain sorted bam files, containing aligned reads, and subsequently pile up format files. VarScan v2.3.6 (Koboldt et al. 2012) was then used to identify SNPs (parameters: p-value 0.1, min-coverage 20 --min-reads2 15 --min-avg-qual 30 --min-var-freq 0.9). A different set of parameters was subsequently used to account for the clones with lower coverage (with modified min-coverage of 10 and --min-reads2 of 6). For clones where no SNP was identified in the *trlG* gene automatically, manual inspection of the alignment files followed by PCR and Sanger sequencing were used to check for the presence of any insertions/deletions. In particular, to sequence the *trlG* gene in the JC and JD strains as well as the tolerant clones S15 and S29, either the gene was amplified using primer pair 00323SecF/00323SecR or the entire operon was amplified using recOSeqF/reOSeqR and the resulting PCR products were purified and sent for Sanger sequencing. All primer sequences used in this study are listed in Table 2.

A prediction of the protein structure of TrlG was performed using I-TASSER (Y. Zhang 2008) with default parameters. The location of the amino acid substitutions on the protein was visualised using UCSF Chimera (Pettersen et al. 2004).

To investigate the presence of the gene in other bacteria, *trlG* was translated using the standard codon usage and the amino acid sequence was used as input for BLASTP v2.8.1 against the NCBI protein reference sequences using default search parameters. Hits were retained if they showed at least 60 % sequence identity over 90 % of the query length. Multiple alignments were created using Clustal Omega (Sievers et al.

2011). The Clustal alignment file was then used to infer a maximum likelihood phylogeny using IQ-TREE v 1.6.3 (Nguyen et al. 2015) with the JTT substitution model selected by ModelFinder (Kalyaanamoorthy et al. 2017), and ultrafast bootstrap (Hoang et al. 2018). To reconstruct the *Photorhabdus recA* phylogeny, *recA* nucleotide sequences were obtained from the *Photorhabdus* genomes present in the NCBI reference genomic sequence database and analysed using the Phylogeny.fr platform. The sequences were aligned using MUSCLE and ambiguity was removed using Gblocks. A maximum likelihood tree was then constructed using PhyML and 500 bootstrap iterations were performed. Phylogenetic trees were visualised using the Interactive Tree Of Life (iTOL) (Letunic and Bork 2019). For MultiGeneBlast analysis (Medema, Takano, and Breitling 2013), a database was created from the bacterial Genbank division and a homology search was performed with default parameters, with the three TRL genes as input.

4.4 Construction of a Δtrl mutant

A markerless Δtrl deletion mutant was created by double homologous recombination using the suicide plasmid pDS132 (Philippe et al. 2004). Regions upstream and downstream of the TRL were amplified using primer pairs DrecOUF/DrecOUR and DrecODF/DrecODR respectively. The purified PCR products were mixed and used as a template in a second joining PCR with primers DrecOUF/DrecODR. The resulting product was digested using enzymes SacI and PstI and ligated into digested and dephosphorylated pDS132. The resulting plasmid was introduced by transformation into the donor *E. coli* strain S17.1 lamda-pir. For conjugations into *Photorhabdus*, the rifampicin resistant *P. luminescens* DJC strain was used as a recipient. Briefly, exponentially growing cultures of *P. laumondii* DJC Rif^r in LB containing 1 mM MgCl₂ and *E. coli* S17.1 lamda-pir harbouring pDS132(Δtrl) were harvested and combined at a ratio 4:1 of acceptor to donor bacteria. Matings were spotted on LBA plates containing 1 mM MgCl₂ and incubated overnight at 28 °C. Bacteria were then harvested and aliquots were plated on LBA supplemented with 15 µg/ml chloramphenicol and 50 µg/ml rifampicin. Possible transconjugants and hence first recombinants were confirmed by PCR using primer pairs TestRecO1/00323SeqR and polA3F'/ TestRecG4. Successful first recombinants were grown in LB overnight and

then the culture was diluted in fresh LB and grown to mid exponential. At this point serial dilutions were made and aliquots were plated on LBA supplemented with 0.2 % sucrose and incubated at 28 °C. The resulting colonies were tested for deletion of the TRL operon by PCR using primers TestRecO1/TestRecG4.

4.5 Complementation

For complementation, the entire TRL was amplified using primers recOFEcoRI/recORSpeI and the resulting PCR product was digested with EcoRI and SpeI and ligated into a modified version of vector pBCSK (that had been previously digested with AseI and KpnI to remove Plac). The resulting plasmid, termed pBCSK'(TRL), and the empty pBCSK' were introduced separately into the Δtrl deletion mutant or into SNP mutants by transformation. To prepare cells for transformation, bacteria growing exponentially in 100 ml LB were harvested at an OD₆₀₀ of 0.2-0.3 and kept for 90 min on ice. They were then collected by centrifugation and washed twice with ice-cold SH buffer at pH 7 (10 mM HEPES containing 5 % w/v sucrose). The cells were resuspended in 160 µl SH buffer and 50 µl aliquots were used for electroporation with the following conditions: 2.5 kV, 25 µF and 200 Ω in 2 mm cuvettes. This was followed by addition of 1 ml LB and incubation at 28°C, 180 rpm for 3 h. Aliquots were plated on LB agar supplemented with 25 µg/ml chloramphenicol. Colonies were tested for the presence of the plasmid by PCR using primers M13R/Seq03223F.

4.6 Infection assays

The infections were performed using 5th instar larvae of *Galleria mellonella*. The bacteria were grown in LB at 28 °C and then collected and resuspended in PBS to an OD₆₀₀ of 1.5. The suspension was then serially diluted to obtain a final suspension with an infection dose of ~50 colony forming units per 10 µl. The *Galleria* larvae were infected by injection through the first proleg and incubated either at room temperature or 36 °C. For the experiment conducted at room temperature two groups of ten larvae were infected per strain, while for the experiment at 36 °C, 8 larvae were infected per strain. The experiment was conducted twice.

496

497 **4.7 Construction of GFP promoter fusions**

498 The TRL promoter regions were amplified using Q5 DNA polymerase and with *P.*
 499 *laumondii* DJC DNA as a template and using the following primer pairs:
 500 Prec2F/PrecR for promoter region PA, Prec2F/Prev00325 for promoter region PB,
 501 Prec1f/Prev00325 for promoter region PC and Prec3F/Prev00325 for promoter region
 502 PD. PCR products were digested using BamHI and SalI and purified. They were then
 503 ligated into the broad range vector pGA-G1, which carries a promoterless *gfp*-mut3
 504 (Leo Eberl, University of Zurich). The ligations were introduced by transformation
 505 into *E. coli* DH5a lamda-pir. Successful transformants were confirmed by PCR and
 506 plasmids were isolated using the Qiagen miniprep kit and introduced into *E. coli*
 507 strain S17.1 lamda pir to be used as a donor in conjugation, which was performed as
 508 described above.

509

510 **4.8 Growth and fluorescence measurements of reporter strains**

511 *P. laumondii* DJC strains harbouring the reporter constructs were grown overnight in
 512 LB supplemented with kanamycin at 28 °C and the cultures were then diluted to fresh
 513 media to an OD600 of ~0.05. The diluted cultures were aliquoted in black flat-
 514 bottomed µClear 96-well plates (CELLSTAR, Greiner) and placed in an Omega
 515 Fluostar (BMG Labtech) microplate reader. They were incubated at 28 °C with double
 516 orbital shaking at 300 rpm. Readings of absorbance at 600 nm as well as GFP
 517 fluorescence (gain 1000) were taken every 30 min. For the temperature shift
 518 experiment, after 5 h of growth at 28 °C the temperature of the microplate reader was
 519 increased to 36 °C (this took place in ~ 2 min). Then readings were taken immediately
 520 following the shift and at 30 min intervals after that.

521

522 **5 Acknowledgements**

523 We would like to thanks Prof. Mark Pallen for the use of his MiSeq machine.

524

525 6 Conflict of Interest

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

528

529 7 Author Contributions

530 AH and NW designed the study; AH, JH and GM performed the mutant isolation
531 experiments; JH performed the structural prediction studies; AH performed the
532 remaining experiments and analysed the data. AH and NW wrote the initial
533 manuscript draft. GM reviewed the manuscript. All authors have approved the final
534 manuscript.

535

536 8 Funding

537 This work was funded by Warwick Medical School, University of Warwick, the
538 Leverhulme Trust grant RPG-2015-194 and the EPSRC MOAC Centre for Doctoral
539 Training (DTPEP/F500378/1).

540

541 9 Tables

542 **Table 1** TrlG amino acid substitutions that result from the SNPs identified in the
543 sequenced isolates, as well as isolates JA and JB. The isolates are separated based on
544 the experiment and thus the lineage they originate from. Note that in experiment 3
545 two independent starting cultures were used. *Inspection of the aligned sequencing
546 reads for clone S29 revealed possible SNPs or small insertions/deletions at the 3' of
547 *trlG*, however it was not possible to confirm that with Sanger sequencing of the
548 amplified region.

Experiment	Clone
(lineage)	TrlG Amino acid substitution

1	S1	Ser16 --> Asn
	S2	n/a
	S3	n/a
	S4	Arg126 --> Gln
	S5	n/a
	S6	Arg80 --> His
	S7	Arg80 --> His
	S8	n/a
	S9	n/a
	S10	n/a
2	S11	Ser208 --> STOP
	S12	Ala193 --> Asp
	S13	Gly354 --> Val
	S14	Ala193 --> Asp
	S15	n/a (IS transposase insertion)
	S16	Gly354 --> Val
	S17	Gly354 --> Val
	S18	Ser331 --> Tyr
	S19	n/a
	S20	Ser331--> Tyr
	S21	n/a

	S22	Ala75 --> Thr
3(a)	S23	Val35 --> Ile
	S24	Pro318--> Arg
	S25	Ala36 --> Val
	S26	n/a
	S27	Asn335 --> Asp
3(b)	S28	Gln249 --> STOP
	S29	n/a*
	S30	Gln249 --> STOP
	JA	Pro318--> Leu
	JB	n/a (IS transposase insertion)

549

550

551 **Table 2:** Oligonucleotide primers used in this study. The positions of restriction sites
552 are shown underlined.

Name	Sequence (5'-3') and restriction enzyme site
polA 3'F	gggattgtctgctatctgag
dsbA 3'F	ggattatgatgctgctatgag
DrecOUF	aatgagctcgacgataaagggtatgaggag, SacI
DrecOUR	gaagtgattggagtcacatatttaacactactc
DrecODF	gagtagtgtaaataatgtgactccaaatcacttc

DrecODR	ttactgcagattgaaatgccggtagc, PstI
TestRecO1	atacaccatacatggctcc
00323SeqF	tggaaggtggtgcagaag
00323SeqR	gcgacagtctgattgctc
TestRecG4	ttatggcatttagcgtatctg
Prec1F	ataggatccactgtcatattgccttagc, BamHI
Prec2F	accggatccaaatcttcttcattatctg, BamHI
Prec3F	aaaggatccaaacgagatttacagacg, BamHI
PrecR	ttagtcgacatttaacactactcacgtgc, SalI
Prev00325	ttagtcgacctcattaataccctctc, SalI

553 10 Figure Legends

554

555 **Figure 1.** A) Genetic organisation of the Temperature restriction locus. B) Domain
556 architecture of TrlG and the presence of substitution mutations in the temperature
557 tolerant clones. Vertical lines indicate the positions of the mutations; red depicts stop
558 mutations. The coloured boxes show the domains identified by Pfam; Magenta:
559 Alba_2 (Pfam domain PF04326, at amino acid positions 14-128) and light blue:
560 HATPase_c_4 (Pfam domain PF13749, at amino acid positions 298-365). C) **(Left)** I-
561 TASSER simulation of TrlG with the distribution of mutations mapped. Orange
562 indicates SNP positions, whilst red depicts stop mutations. **(Right)** The same model
563 as in (Left) with homology domains mapped in colour. Images prepared with UCSF
564 Chimera.

565

566 **Figure 2.** A) Growth of the 30 sequenced tolerant clones and *P. laumondii* DJC (WT)
567 on LB agar at 28 °C and 36 °C. B) Growth of the additional two clones isolated
568 independently (JA and JB) compared to *P. laumondii* DJC (WT) as well as of the Δtrl
569 deletion strain compared to the isogenic rifampicin resistant *P. laumondii* DJC
570 (DJCRif) at 28 °C and 36 °C.

571

572 **Figure 3.** Growth of the Δtrl deletion mutant complemented with either the empty
573 pBCSK' vector or pBCSK' carrying the TRL operon on A) LB agar and B) liquid LB
574 media at 28 °C and 36 °C. The asterisk denotes significant difference at $p_{adj} < 0.05$.
575 Results show the averages and standard error from four biological replicates.

576

577 **Figure 4.** Virulence of the *trl* mutants. The results show the number of surviving
578 *Galleria mellonella* larvae infected with either the WT strain, the S1 SNP mutant, the
579 RifR WT strain or the Δtrl deletion mutant at different time points. *G. mellonella*
580 larvae injected with PBS were used as control. Twenty larvae were used for each
581 strain and the experiment was repeated with similar results.

582

583 **Figure 5.** Up-regulation of the promoter of the TRL operon upon shift to 36 °C. A)
584 Schematic diagram showing the different regions used in the promoter reporter
585 constructs. The expanded panel shows the region between *trlF* (*PluDJC_RS01880*)
586 and *polA* with the predicted promoters based on BPROM indicated as arrows on top
587 of the sequence. At the bottom are the regions amplified and introduced separately
588 into pGAG1 upstream of the *gfpmut3* gene to create promoter constructs P_AGFP-
589 P_DGFP. The diagrams are color coded to reflect the colors of the data plotted below.
590 B) Left: Fluorescence of the *P. laumondii* DJC reporter strains following a shift in
591 temperature from 28 °C to 36 °C after 5 h of growth as indicated by the arrow. Right:
592 Fluorescence of the *P. laumondii* DJC reporter strains grown continuously at 28 °C.
593 Results are arbitrary fluorescence units following a subtraction of the fluorescence of

the *P. laumondii* DJC carrying pGAG1 in the absence of any promoter to account for any autofluorescence and they represent the mean of three biological replicates +/- standard error. C) The rate of change in fluorescence in *P. laumondii* DJC carrying pGAG1(PCGFP), calculated by subtracting the fluorescence at a given time point by the fluorescence of the previous time point; left: following a shift in temperature from 28 °C to 36 °C after 5 h of growth as indicated by the arrow; right: at 28 °C. Results represent the mean of three biological replicates +/- standard error.

Figure 6. Maximum likelihood tree constructed using the *Photorhabdus recA* nucleotide sequences from RefSeq genomes. Black circles indicate branch support over 80 %. Highlighted are the strains that were found to contain a copy of the *trlG* gene.

Figure 7. Maximum likelihood tree constructed using protein sequences with over 60 % sequence identity to the *Photorhabdus laumondii* TrlG protein (with a query cover > 90 %). Nodes with support values higher than 80 % are indicated with black circles. The external coloured ring represents different genera. Protein identifiers for all the entries can be found in Supplementary table 1.

Figure 8. Conservation and genetic organization of the TRL operon in representative species as detected by Multigene blast. In red is *trlG*, in green is *trlF* and in blue is *trlR*; genes coloured identically in other organisms denote the identified homologues of *trlG*, *trlF* and *trlR* respectively.

11 Supplementary material

Supplementary Figures 1-4

Supplementary Table 1

12 Data Availability Statement

The raw sequencing data generated and analysed in this study has been deposited in the NCBI database under BioProject ID PRJNA596169, while raw sequencing reads for the WT isolate in our lab can be found in the short-read archive (SRA) using accession number SRR8307122. The reference genome sequence for *P. luminescence* subsp *laumondii* DJC is available on NCBI RefSeq with the accession number NZ_CP024900.1.

13 References

- Abu Kwaik, Yousef, and Dirk Bumann. 2013. "Microbial Quest for Food in Vivo: 'Nutritional Virulence' as an Emerging Paradigm." *Cellular Microbiology* 15 (6): 882–90. <https://doi.org/10.1111/cmi.12138>.
- Bager, Ragnhild, Mohammad Roghanian, Kenn Gerdes, and David J. Clarke. 2016. "Alarmon (p)PpGpp Regulates the Transition from Pathogenicity to Mutualism in *Photobacterium luminescens*." *Molecular Microbiology* 100 (4): 735–47. <https://doi.org/10.1111/mmi.13345>.
- Boemare, Noel, and Raymond Akhurst. 1988. "Biochemical and Physiological Characterization of Colony Form Variants in *Xenorhabdus* Spp. (Enterobacteriaceae)." *Microbiology* 134 (3): 751–61. <https://doi.org/10.1099/00221287-134-3-751>.
- Ciche, Todd A., and Jerald C. Ensign. 2003. "For the Insect Pathogen *Photobacterium luminescens*, Which End of a Nematode Is Out?" *Applied and Environmental Microbiology* 69 (4): 1890–97. <https://doi.org/10.1128/AEM.69.4.1890-1897.2003>.
- Duchaud, Eric, Christophe Rusniok, Lionel Frangeul, Carmen Buchrieser, Alain Givaudan, Séad Taourit, Stéphanie Bocs, et al. 2003. "The Genome Sequence of the Entomopathogenic Bacterium *Photobacterium luminescens*." *Nature Biotechnology* 21 (11): 1307–13. <https://doi.org/10.1038/nbt886>.

- 651 Dutta, Ankhi, Anthony R. Flores, Paula A. Revell, and Lisa Owens. 2018. “Neonatal
652 Bacteremia and Cutaneous Lesions Caused by *Photorhabdus Luminescens*: A
653 Rare Gram-Negative Bioluminescent Bacterium.” *Journal of the Pediatric
654 Infectious Diseases Society* 7 (3): e182–84. <https://doi.org/10.1093/jpids/piy064>.
- 655 Fischer-Le Saux, M, V Viallard, B Brunel, Phillippe Normand, and N E Boemare.
656 1999. “Polyphasic Classification of the Genus *Photorhabdus* and Proposal of
657 New Taxa: *P. Luminescens* Subsp. *Luminescens* Subsp. Nov., *P. Luminescens*
658 Subsp. *Akhurstii* Subsp. Nov., *P. Luminescens* Subsp. *Laumondii* Subsp. Nov.,
659 *P. Temperata* Sp. Nov., *P. Temperata* Subs.” *International Journal of Systematic
660 Bacteriology* 49 (4): 1645–56. <https://doi.org/10.1099/00207713-49-4-1645>.
- 661 Gerrard, John G, and Robert P Stevens. 2017. “A Review of Clinical Cases of
662 Infection with *Photorhabdus Asymbiotica*.” In *The Molecular Biology of
663 Photorhabdus Bacteria*, edited by Richard H ffrench-Constant, 179–91. Cham:
664 Springer International Publishing. https://doi.org/10.1007/82_2016_56.
- 665 Guijarro, José A., Desirée Cascales, Ana I. García-Torrico, Mario García-Domínguez,
666 and Jessica Méndez. 2015. “Temperature-Dependent Expression of Virulence
667 Genes in Fish-Pathogenic Bacteria.” *Frontiers in Microbiology* 6 (JUL): 1–11.
668 <https://doi.org/10.3389/fmicb.2015.00700>.
- 669 Hoang, Diep Thi, Olga Chernomor, Arndt von Haeseler, Bui Quang Minh, and Le Sy
670 Vinh. 2018. “UFBoot2: Improving the Ultrafast Bootstrap Approximation.”
671 *Molecular Biology and Evolution* 35 (2): 518–22.
672 <https://doi.org/10.1093/molbev/msx281>.
- 673 Ishioka, Ken, Hiroshi Iwasaki, and Hideo Shinagawa. 1997. “Roles of the RecG Gene
674 Product of *Escherichia Coli* in Recombination Repair: Effects of the
675 .DELTA.RecG Mutation on Cell Division and Chromosome Partition.” *Genes &
676 Genetic Systems* 72 (2): 91–99. <https://doi.org/10.1266/ggs.72.91>.
- 677 Jones, Robert T, Maria Sanchez-Contreras, Isabella Vlisidou, Matthew R Amos,
678 Guowei Yang, Xavier Muñoz-Berbel, Abhishek Upadhyay, et al. 2010.
679 “*Photorhabdus* Adhesion Modification Protein (Pam) Binds Extracellular
680 Polysaccharide and Alters Bacterial Attachment.” *BMC Microbiology* 10 (1):

- 681 141. <https://doi.org/10.1186/1471-2180-10-141>.
- 682 Kalyaanamoorthy, Subha, Bui Quang Minh, Thomas K F Wong, Arndt von Haeseler,
683 and Lars S Jermiin. 2017. “ModelFinder: Fast Model Selection for Accurate
684 Phylogenetic Estimates.” *Nature Methods* 14 (6): 587–89.
685 <https://doi.org/10.1038/nmeth.4285>.
- 686 Kazimierczak, Waldemar, Henryk Skrzypek, Ewa Sajnaga, Marcin Skowronek, Adam
687 Waśko, and Anna Kreft. 2017. “Strains of Photorhabdus Spp. Associated with
688 Polish Heterorhabditis Isolates: Their Molecular and Phenotypic
689 Characterization and Symbiont Exchange.” *Archives of Microbiology* 199 (7):
690 979–89. <https://doi.org/10.1007/s00203-017-1368-z>.
- 691 Koboldt, Daniel C, Qunyu Zhang, David E Larson, Dong Shen, Michael D
692 Mclellan, Ling Lin, Christopher A Miller, Elaine R Mardis, Li Ding, and Richard
693 K Wilson. 2012. “VarScan 2 : Somatic Mutation and Copy Number Alteration
694 Discovery in Cancer by Exome Sequencing,” 568–76.
695 <https://doi.org/10.1101/gr.129684.111.568>.
- 696 Kumar, Gokhlesh, Simon Menanteau-Ledouble, Mona Saleh, and Mansour El-
697 Matbouli. 2015. “Yersinia Ruckeri, the Causative Agent of Enteric Redmouth
698 Disease in Fish.” *Veterinary Research* 46 (1): 1–10.
699 <https://doi.org/10.1186/s13567-015-0238-4>.
- 700 Lages, Marta A., Miguel Balado, and Manuel L. Lemos. 2019. “The Expression of
701 Virulence Factors in Vibrio Anguillarum Is Dually Regulated by Iron Levels and
702 Temperature.” *Frontiers in Microbiology* 10 (October).
703 <https://doi.org/10.3389/fmicb.2019.02335>.
- 704 Langmead, Ben, and Steven L Salzberg. 2012. “Fast Gapped-Read Alignment with
705 Bowtie 2.” *Nat Methods* 9 (4): 357–59. <https://doi.org/10.1038/nmeth.1923>.
- 706 Lango, Lea, and David J Clarke. 2010. “A Metabolic Switch Is Involved in Lifestyle
707 Decisions in Photorhabdus Luminescens.” *Molecular Microbiology* 77 (6):
708 1394–1405. <https://doi.org/10.1111/j.1365-2958.2010.07300.x>.
- 709 Lease, Richard A, and Sarah A Woodson. 2004. “Cycling of the Sm-like Protein Hfq

- 710 on the DsrA Small Regulatory RNA.” *Journal of Molecular Biology* 344 (5):
711 1211–23. <https://doi.org/10.1016/j.jmb.2004.10.006>.
- 712 Letunic, Ivica, and Peer Bork. 2019. “Interactive Tree Of Life (ITOL) v4: Recent
713 Updates and New Developments.” *Nucleic Acids Research* 47 (W1): W256–59.
714 <https://doi.org/10.1093/nar/gkz239>.
- 715 Li, Heng, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor
716 Marth, Goncalo Abecasis, and Richard Durbin. 2009. “The Sequence
717 Alignment/Map Format and SAMtools.” *Bioinformatics* 25 (16): 2078–79.
718 <https://doi.org/10.1093/bioinformatics/btp352>.
- 719 Machado, Ricardo A. R., Daniel Wüthrich, Peter Kuhnert, Carla C. M. Arce, Lisa
720 Thönen, Celia Ruiz, Xi Zhang, et al. 2018. “Whole-Genome-Based Revisit of
721 *Photorhabdus* Phylogeny: Proposal for the Elevation of Most *Photorhabdus*
722 Subspecies to the Species Level and Description of One Novel Species
723 *Photorhabdus Bodei* Sp. Nov., and One Novel Subspecies *Photorhabdus*
724 *Laumondii* Subsp. .” *International Journal of Systematic and Evolutionary*
725 *Microbiology* 68 (8): 2664–81. <https://doi.org/10.1099/ijsem.0.002820>.
- 726 Maher, Abigail M D, Mohamed A M Asaiyah, Caroline Brophy, and Christine T
727 Griffin. 2017. “An Entomopathogenic Nematode Extends Its Niche by
728 Associating with Different Symbionts.” *Microbial Ecology* 73 (1): 211–23.
729 <https://doi.org/10.1007/s00248-016-0829-2>.
- 730 Maneesakorn, Patchareewan, Ruisheng An, Hannah Daneshvar, Kara Taylor,
731 Xiaodong Bai, Byron J. Adams, Parwinder S. Grewal, and Angsumarn
732 Chandrapatya. 2011. “Phylogenetic and Cophylogenetic Relationships of
733 Entomopathogenic Nematodes (*Heterorhabditis*: *Rhabditida*) and Their
734 Symbiotic Bacteria (*Photorhabdus*: *Enterobacteriaceae*).” *Molecular*
735 *Phylogenetics and Evolution* 59 (2): 271–80.
736 <https://doi.org/10.1016/j.ympev.2011.02.012>.
- 737 Mathur, Chetna, Jyoti Kushwah, Vishal S. Somvanshi, and Tushar K. Dutta. 2018. “A
738 37 KDa Txp40 Protein Characterized from *Photorhabdus Luminescens* Sub Sp.
739 *Akhurstii* Conferred Injectable and Oral Toxicity to Greater Wax Moth, *Galleria*

- 740 Mellonella.” *Toxicon* 154 (November): 69–73.
 741 <https://doi.org/10.1016/j.toxicon.2018.09.007>.
- 742 Medema, Marnix H., Eriko Takano, and Rainer Breitling. 2013. “Detecting Sequence
 743 Homology at the Gene Cluster Level with Multigeneblast.” *Molecular Biology*
 744 *and Evolution* 30 (5): 1218–23. <https://doi.org/10.1093/molbev/mst025>.
- 745 Mendez, Jessica, Desirée Cascales, Ana I Garcia-Torrico, and Jose A Guijarro. 2018.
 746 “Temperature-Dependent Gene Expression in *Yersinia Ruckeri*: Tracking
 747 Specific Genes by Bioluminescence During in Vivo Colonization.” *Frontiers in*
 748 *Microbiology* 9: 1098. <https://doi.org/10.3389/fmicb.2018.01098>.
- 749 Møller, Thorleif, Thomas Franch, Peter Højrup, Douglas R Keene, Hans Peter
 750 Bächinger, Richard G Brennan, and Poul Valentin-Hansen. 2002. “Hfq: A
 751 Bacterial Sm-like Protein That Mediates RNA-RNA Interaction.” *Molecular Cell*
 752 9 (1): 23–30. [https://doi.org/10.1016/s1097-2765\(01\)00436-1](https://doi.org/10.1016/s1097-2765(01)00436-1).
- 753 Mulley, Geraldine, Michael L. Beeton, Paul Wilkinson, Isabella Vlisidou, Nina
 754 Ockendon-Powell, Alexia Hapeshi, Nick J. Tobias, et al. 2015. “From Insect to
 755 Man: *Photobacterium* Sheds Light on the Emergence of Human Pathogenicity.”
 756 Edited by Mikael Skurnik. *PLOS ONE* 10 (12): e0144937.
 757 <https://doi.org/10.1371/journal.pone.0144937>.
- 758 Nguyen, Lam Tung, Heiko A. Schmidt, Arndt Von Haeseler, and Bui Quang Minh.
 759 2015. “IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating
 760 Maximum-Likelihood Phylogenies.” *Molecular Biology and Evolution* 32 (1):
 761 268–74. <https://doi.org/10.1093/molbev/msu300>.
- 762 Pettersen, Eric F., Thomas D. Goddard, Conrad C. Huang, Gregory S. Couch, Daniel
 763 M. Greenblatt, Elaine C. Meng, and Thomas E. Ferrin. 2004. “UCSF Chimera -
 764 A Visualization System for Exploratory Research and Analysis.” *Journal of*
 765 *Computational Chemistry*. <https://doi.org/10.1002/jcc.20084>.
- 766 Philippe, Nadège, Jean Pierre Alcaraz, Evelyne Coursange, Johannes Geiselmann,
 767 and Dominique Schneider. 2004. “Improvement of PCVD442, a Suicide Plasmid
 768 for Gene Allele Exchange in Bacteria.” *Plasmid* 51 (3): 246–55.

769 <https://doi.org/10.1016/j.plasmid.2004.02.003>.

770 Rudolph, Christian J, Akeel a Mahdi, Amy L Upton, and Robert G Lloyd. 2010.
771 “RecG Protein and Single-Strand DNA Exonucleases Avoid Cell Lethality
772 Associated with PriA Helicase Activity in Escherichia Coli.” *Genetics* 186 (2):
773 473–92. <https://doi.org/10.1534/genetics.110.120691>.

774 Sievers, Fabian, Andreas Wilm, David Dineen, Toby J Gibson, Kevin Karplus,
775 Weizhong Li, Rodrigo Lopez, et al. 2011. “Fast , Scalable Generation of High-
776 Quality Protein Multiple Sequence Alignments Using Clustal Omega,” no. 539.
777 <https://doi.org/10.1038/msb.2011.75>.

778 Solovyev, Victor, and Asaf Salamov. 2011. “Automatic Annotation of Microbial
779 Genomes and Metagenomic Sequences.” In *Metagenomics and Its Applications*
780 *in Agriculture, Biomedicine and Environmental Studies*.

781 Somvanshi, V. S., R. E. Sloup, J. M. Crawford, A. R. Martin, A. J. Heidt, K.-s. Kim,
782 J. Clardy, and T. A. Ciche. 2012. “A Single Promoter Inversion Switches
783 Photorhabdus Between Pathogenic and Mutualistic States.” *Science* 337 (6090):
784 88–93. <https://doi.org/10.1126/science.1216641>.

785 Tailliez, P., C. Laroui, N. Ginibre, A. Paule, S. Pages, and N. Boemare. 2010.
786 “Phylogeny of Photorhabdus and Xenorhabdus Based on Universally Conserved
787 Protein-Coding Sequences and Implications for the Taxonomy of These Two
788 Genera. Proposal of New Taxa: X. Vietnamensis Sp. Nov., P. Luminescens
789 Subsp. Caribbeanensis Subsp. Nov., P. L.” *INTERNATIONAL JOURNAL OF*
790 *SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY* 60 (8): 1921–37.
791 <https://doi.org/10.1099/ijms.0.014308-0>.

792 Tobias, Nicholas J., Antje K. Heinrich, Helena Eresmann, Patrick R. Wright, Nick
793 Neubacher, Rolf Backofen, and Helge B. Bode. 2017. “Photorhabdus-Nematode
794 Symbiosis Is Dependent on Hfq-Mediated Regulation of Secondary
795 Metabolites.” *Environmental Microbiology* 19 (1): 119–29.
796 <https://doi.org/10.1111/1462-2920.13502>.

797 Vernikos, Georgios S, and Julian Parkhill. 2006. “Interpolated Variable Order Motifs

798 for Identification of Horizontally Acquired DNA: Revisiting the Salmonella
799 Pathogenicity Islands.” *Bioinformatics (Oxford, England)* 22 (18): 2196–2203.
800 <https://doi.org/10.1093/bioinformatics/btl369>.

801 Waterfield, Nick R., Todd Ciche, and David Clarke. 2009. “*Photorhabdus* and a Host
802 of Hosts.” *Annual Review of Microbiology* 63 (1): 557–74.
803 <https://doi.org/10.1146/annurev.micro.091208.073507>.

804 Zamora-Lagos, Maria-Antonia, Simone Eckstein, Angela Langer, Athanasios
805 Gazanis, Friedhelm Pfeiffer, Bianca Habermann, and Ralf Heermann. 2018.
806 “Phenotypic and Genomic Comparison of *Photorhabdus Luminescens* Subsp.
807 *Laumondii* TT01 and a Widely Used Rifampicin-Resistant *Photorhabdus*
808 *Luminescens* Laboratory Strain.” *BMC Genomics* 19 (1): 854.
809 <https://doi.org/10.1186/s12864-018-5121-z>.

810 Zhang, A., S Altuvia, A Tiwari, L Argaman, R Hengge-Aronis, and G Storz. 1998.
811 “The OxyS Regulatory RNA Represses RpoS Translation and Binds the Hfq
812 (HF-I) Protein.” *The EMBO Journal* 17 (20): 6061–68.
813 <https://doi.org/10.1093/emboj/17.20.6061>.

814 Zhang, Yang. 2008. “I-TASSER Server for Protein 3D Structure Prediction.” *BMC*
815 *Bioinformatics*. <https://doi.org/10.1186/1471-2105-9-40>.

816

Figure 1

A

B

C

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Tree scale: 0.1

Figure 7

Tree scale: 0.1

Figure 8

Photorhabdus laumondii DJC

Photorhabdus laumondii TTO1

Vibrio anguillarum VIB43

Yersinia ruckeri PB-H2, plasmid : pYR3.

Oblitimonas alkaliphila D2441