Discovery of a Highly Virulent Strain of *Photorhabdus luminescens* ssp. *akhurstii* from Meghalaya, India

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Abstract *Photorhabdus* is an insect-pathogenic Gram negative enterobacterium found in the gut of *Heterorhabditis* nematodes. *Photorhabdus* is highly virulent to insects, and can kill insects rapidly upon injection at very low concentrations of one to few cells. We characterized the virulence of *Photorhabdus* symbionts isolated from the *Heterorhabditis* nematodes collected from various parts of India by injecting different concentrations of bacterial cells into fourth instar larval stage of insect *Galleria mellonella*. *Photorhabdus luminescens* ssp. *akhurstii* strain IARI-SGMG3 from Meghalaya was identified as the most virulent of all the tested strains on the basis of LT₅₀ and LC₅₀ values. This study forms a basis for further investigations on the genetic basis of virulence in *Photorhabdus* bacteria.

Keywords *Photorhabdus* · Injectable toxicity · Virulence · *Heterorhabditis* · *Galleria* · Meghalaya

Microbes like bacteria, fungi, viruses, nematodes and protists are known insect pathogens, and are replacing chemicals for agricultural insect-pest management [1, 2]. Insect-parasitic nematodes belonging to families Heterorhabditidae and Steinernematidae are commonly used for the control of insects [3]. These nematodes live in

a symbiotic relationship with the Gram-negative enterobacteria of the genus *Photorhabdus* and *Xenorhabdus*, respectively. *Photorhabdus* and *Xenorhabdus* bacteria display a contrasting life cycle; they are nematode symbionts but virulent pathogen of insects [4, 5]. In nature, the infective juvenile (IJ) stage of these entomopathogenic nematodes (EPNs) is found in soil and carries the bacteria in their guts. The IJs parasitize the insects, enter the insect haemocoel and regurgitate their symbiont bacteria. The bacteria multiply in the haemocoel and kill the insect by causing septicemia and production of many secondary metabolites and a multitude of toxins [6, 7].

Xenorhabdus and *Photorhabdus* produce a range of toxins and secondary metabolites [8, 9]. *Photorhabdus* has received much commercial attention as a new biocontrol agent because of high pathogenicity towards a wide variety of insects—just a few cells of the bacterium are capable of killing an insect caterpillar [6, 7]. The complete *P. luminescens* ssp. *laumondii* genome revealed presence of a large repertoire of genes coding for hemolysins, adhesions, lipases and proteases, toxins and wide range of antibiotic synthesizing genes. *Photorhabdus* genome consists of 23 biosynthetic gene clusters, and approximately 6.5 % of the *Photorhabdus* genome codes for secondary metabolites [8, 10]. There has been some interest in the utilization of *Photorhabdus* toxin genes for generating transgenic plants for insect resistance [11, 12]. In addition, several studies establish this bacterium as a standalone insecticide; as foliar application [13, 14], or in the form of alginate beads [15].

The genus *Photorhabdus* consists of four species—*P. asymbiotica*, *P. luminescens*, *P. heterorhabditis* and *P. temperata*. All *Photorhabdus* are nematode symbiont and are safe for humans, except *P. asymbiotica* which was reported as a human pathogen in USA and Australia [16].

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Newer variants of toxins are expected to be discovered in the new bacterial isolates; therefore each new strain could potentially possess a new and hyper- or hypo-toxic variant of the toxin(s). Previously, we isolated six strains of insect-parasitic nematode *Heterorhabditis* spp. from various agro-climatic zones of India and identified the most virulent nematode strain(s) [17, 18]. Here we quantitate the virulence of various native *Photorhabdus* isolates on *Galleria mellonella* to prepare groundwork for the study of genetic basis of virulence in *Photorhabdus*.

Details of bacterial strains and their nematode hosts are given in Supplementary Table 1. The nematodes were maintained in Division of Nematology, ICAR-IARI, New Delhi, on the fourth instar larval stage of *G. mellonella* in the laboratory using standard procedures. Isolation of symbiont bacterial strains was done as per standard procedure. Freshly harvested IJs were surface sterilized and crushed by motorized tissue grinder in a microcentrifuge tube. The crushed homogenate was streaked on NBTA (in 1 l water: beef extract 3 g, peptone 5 g, bromothymol blue 0.025 g, NaCl 4 g, sodium pyruvate 1 g, agar 15 g, and 2, 3, 5- triphenyl tetrazolium chloride 0.04 g) media, pH-7.2. The plates were incubated for 48 h at 28 °C. Green bioluminescent primary colonies from all six isolations were picked and purified. 16S rRNA gene sequence identified strains of bacteria as *P. luminescens*. *P. luminescens* ssp. *laumondii* TTO1 (a gift from Dr. Pavel Hyrs) was used as control. Laboratory cultured fourth instar *G. mellonella* larvae were used for virulence assays. Twelve well polystyrene plates (Cat. No. TPP12, HiMedia laboratories, Mumbai, India) were used for in vitro virulence assays. The fourth-instar *G. mellonella* larvae were surface-sterilized by 70 % alcohol. Individual surface-sterilized larvae was kept in each well in the ten

replicates. Bacterial strains were grown in LB medium supplemented with 1 g/L sodium pyruvate for 48 h at 28 °C with constant shaking at 200 rpm. One hundred µl of this starter culture was inoculated into fresh 5 ml of LBP medium, incubated at 28 °C to achieve exponential phase (OD_{600} –1.00). The cultures were washed, and serially diluted to extinction in normal saline to get 1–20 colony forming units (CFU). Thirty µl of the each dilution was injected below the pro-legs of *Galleria* larvae by Hamilton syringe (26s gauge, 50 µl capacity). Normal saline and LBP broth were injected as two controls. The CFUs were quantitated by plating 30 µl culture from each of the bacterial dilutions. Post-injection, the insects in the 12 well plates were kept at 28 °C and mortality was measured at 12 h interval. Dead insects turned brick red colored and luminesced in a dark room. The experiment was independently repeated thrice. We calculated LC_{50} (median lethal concentration) and LT_{50} (median lethal time) as a measure of bacterial virulence using the SPSS v.21 (IBM Corp., NY, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA), respectively.

The bacterial strains killed all of the *G. mellonella* larvae at 48 h post-injection at all the CFU levels as compared to zero mortality in the untreated controls. The LC_{50} values and *G. mellonella* survival at 12 h and 24 h are presented in Supplementary Fig. 1 and Table 1. At 12 h post infection, all the strains were significantly ($P < 0.0001$) virulent as compared to control. IARI-SGMG3 showed the lowest LC_{50} value (5.54) at 12 h as compared to other strains which were IARI-SGLDK1-11.96; IARI-SGMS1-12.16; TTO1-12.7; IARI-SGGJ2- 22.9; IARI-SGHR2 and IARI-SGHR4-24.6. At 12 h, comparison of means at 5 and 10 CFUs showed that IARI-SGMG3 was significantly ($P < 0.0001$) highly virulent as compared to other strains, whereas at 15 CFU, IARI-

Table 1 Virulence of *Photorhabdus* strains against the fourth-instar larvae of *Galleria mellonella*

Photorhabdus strain	12 h [#] after <i>Photorhabdus</i> injection				24 h after <i>Photorhabdus</i> injection				
	LC ₅₀	Mean % survival of <i>Galleria</i>			LC ₅₀	Mean % survival of <i>Galleria</i>			
		5 CFU	10 CFU	15 CFU		1 CFU	5 CFU	10 CFU	15 CFU
IARI-SGMG3	5.54	43.33 ^D	26.67 ^D	16.67 ^E	0.262	23.33 ^C	6.67 ^D	3.33 ^D	0 ^C
IARI-SGGJ2	22.9	80 ^B	76.67 ^B	60 ^C	0.38	30 ^C	23.33 ^C	13.33 ^{CD}	0 ^C
IARI-SGHR2	24.6	100 ^A	86.67 ^{AB}	76.67 ^B	0.49	26.67 ^C	20 ^{CD}	16.67 ^{BCD}	3.33 ^C
IARI-SGHR4	24.6	100 ^A	90 ^{AB}	83.33 ^B	2.3	43.33 ^{BC}	23.33 ^C	20 ^{BC}	16.67 ^B
IARI-SGMS1	12.16	76.67 ^{BC}	60 ^C	36.67 ^D	2.8	56.67 ^B	26.67 ^C	23.33 ^{BC}	16.67 ^B
IARI-SGLDK1	11.96	63.33 ^C	56.67 ^C	36.67 ^D	1.4	60 ^B	30 ^C	23.33 ^{BC}	3.33 ^C
<i>P. luminescens</i> ssp. <i>laumondii</i> TTO1	12.7	86.67 ^{AB}	76.67 ^B	30 ^{DE}	1.7	56.67 ^B	50 ^B	30 ^B	3.33 ^C
Control	n.d.	100 ^A	100 ^A	100 ^A	n.d.	100 ^A	100 ^A	100 ^A	100 ^A
<i>P</i> value		<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
CV (%)	–	6.30	6.72	10.40	–	14.51	16.50	16.32	19.73
SE (d)	–	4.178	3.934	4.672	–	5.876	4.714	3.823	2.887
Tukeys HSD at 5%	–	14.744	13.881	16.485	–	20.733	16.634	13.521	10.186

[#] h hours after *Photorhabdus* injection, CFU colony forming units, n.d. not defined

SGMG3 and TTO1 were at par (Table 1). At 24 h post infection, IARI-SGMG3 appeared the most virulent bacterial strain showing lowest LC_{50} value (0.262) as compared to other tested bacterial strains (Table 1). All the strains were significantly ($P < 0.0001$) virulent as compared to control. The difference in virulence of various strains was less prominent when compared for the mean survival at 24 h, but IARI-SGMG3 was still amongst the most significantly ($P < 0.0001$) virulent strain (Supplementary Fig. 1 and Table 1). On the basis of median lethal time (LT_{50}) values at 1, 5, 10 and 15 CFUs, the tested bacterial strains could be grouped into three different virulence groups (Fig. 1). The first and most virulent group comprised of IARI-SGMG3 showing LT_{50} of 24 h at 1 CFU and 12 h at 5, 10 and 15 CFU. The second virulent group comprised of strains IARI-SGMS1, IARI-SGLDK1 and TTO1 which exhibited LT_{50} of 36 h at 1 CFU, 24 h at 5 and 10 CFU, and 12 h at 15 CFU. The third virulent group comprised of IARI-SGGJ2, IARI-SGHR2 and IARI-SGHR4 that showed one constant LT_{50} value of 24 h at all the CFUs. Comparative LT_{50} values suggest that IARI-SGMG3 was the quickest in killing the insect (Fig. 1). In summary, the LC_{50} and LT_{50} values at 12 and 24 h showed that IARI-SGMG3 was the most virulent amongst the tested bacterial strains.

The virulence of strains resolved better at lower concentrations and earlier time points. We discovered that the strain IARI-SGMG3 was the most virulent strain against *G. mellonella* as compared to all the other strains. Interestingly, in a previous study, it was found that the nematode host of strain IARI-SGMG3 was the quickest in killing *G. mellonella* as compared to all other tested *Heterorhabditis* strains [18]. Taken together, our results suggest that

virulence of bacterial symbiont is the major contributory factor in the overall virulence of its nematode host, and that the high virulence of *Heterorhabditis* strain HMg3 can be attributed to its bacterial partner. This result also suggests that the differences in virulence of *Photorhabdus* symbionts may be responsible for differences in virulence of their nematode hosts. However, one limitation of our study is that it was done in a 12 well plate under laboratory conditions whereas in nature the insect pathogenesis happens in soil which is a highly complex environment.

The general trend of evolution in genus *Photorhabdus* is of increasing virulence [19]. Sequencing and annotation of the *Photorhabdus* genome revealed several insecticidal toxins which vary in their modes of action; some show oral toxicity, others show injectable toxicity or both [20]. These toxins include toxin complexes (Tc) consisting of *tca*, *tcb*, *tcc* and *tcd* genes, Makes Caterpillar Toxins (MCf) which consist MCf1 and MCf2, Txp40 toxin, PirA/B, *Photorhabdus* insect related binary toxins, *Photorhabdus* virulence cassettes (PVCs), *Photorhabdus* insecticidal toxins (Pt toxins), Hemolysins or Hemagglutinin-Related proteins and many others [20]. However, the existence of so many different toxins in *Photorhabdus* is intriguing. How is the expression of these toxins controlled during insect pathogenesis and nematode symbiosis? Are these toxins specific to different orders of insects? How are they expressed during different developmental stages of the nematode and bacteria? All these questions need to be answered so that we can fully understand the tripartite relationship of insect-nematode-bacteria [21]. In addition, the molecular basis for difference in virulence between different strains and species of *Photorhabdus* is still unknown. In order to answer

Fig. 1 Kaplan-Meier survival curves of *G. mellonella* infected by various *Photorhabdus* strains. The dotted line shows survival in control, whereas the solid black line represents survival after injection

of *Photorhabdus* strains. X axis -time in hours (h), Y axis- percent survival of *G. mellonella*. The LT_{50} values are indicated in each curve at $P < 0.0001$

this question, the genetic variance of the toxins, and whether this variance can make a toxin protein hyper- or hypotoxic must be investigated in addition to the toxin expression patterns. Our study identified *Photorhabdus* ‘virulence contrasts’ which may be genetically probed using genomic or proteomic approaches to answer these questions, as was done for determining host specificity using proteomics approach [22].

In summary, we discovered a highly virulent strain of *P. luminescens* ssp. *akhurstii* from Meghalaya. This strain will be investigated for the presence of variants of already known *Photorhabdus* toxins, and for presence of novel toxins which might be responsible for the higher virulence of this strain.

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Compliance with Ethical Standards

Conflict of interest Authors declare no potential conflict of interest.

Human and Animal Rights No animal and human rights were violated during this study.

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