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ORIGINAL ARTICLE

Proteomic Investigation of *Photorhabdus* Bacteria for Nematode-Host Specificity

Ram Kumar[1,2] · Jyoti Kushwah[1] · Sudershan Ganguly[1] · Veena Garg[3] · Vishal S. Somvanshi[1]

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Abstract Majority of animals form symbiotic relationships with bacteria. Based on the number of bacterial species associating with an animal, these symbiotic associations can be mono-specific, relatively simple (2–25 bacterial species/animal) or highly complex (>10^2–10^3 bacterial species/animal). *Photorhabdus* (family-*Enterobacteriaceae*) forms a mono-specific symbiotic relationship with the entomopathogenic nematode *Heterorhabditis.* This system provides a tractable genetic model for animal-microbe symbiosis studies. Here, we investigated the bacterial factors that may be responsible for governing host specificity between nematode and their symbiont bacteria using proteomics approach. Total protein profiles of *P. luminescens* ssp. *laumondii* (host nematode- *H. bacteriophora*) and *P. luminescens* ssp. *akhurstii* (host nematode- *H. indica*) were compared using 2-D gel electrophoresis, followed by identification of differentially expressed proteins by MALDI-TOF MS. Thirty-nine unique protein spots were identified - 24 from *P. luminescens* ssp. *laumondii* and 15 from *P. luminescens* ssp. *akhurstii.* These included proteins that might be involved in determining host specificity directly (for e.g. pilin FimA, outer membrane protein A), indirectly through effect on bacterial secondary metabolism (for e.g. malate dehydrogenase Mdh, Pyruvate formate-lyase PflA, flavo protein WrbA), or in a yet unknown manner (for e.g. hypothetical proteins, transcription regulators). Further functional validation is needed to establish the role of these bacterial proteins in nematode-host specificity.

Keywords *Photorhabdus* · *Heterorhabditis* · Symbiosis · Host specificity · Proteomics · 2-D gel electrophoresis

Introduction

Animal-microbe relationships are a fact of life. These associations can be simple mono-specific associations (for e.g. nematodes-bacteria symbiosis), relatively simple consortia (2–25 bacterial species in an animal, for e.g. leach gut consortium, insect gut consortium) and highly complex consortia (for e.g. vertebrate guts colonized by >10^2–10^3 species of bacteria) [1]. Symbionts affect the physiology, immunity, metabolism, behaviour, growth and development of host, and offer protection to the host [2, 3]. It is astonishing that trillions of bacteria can live symbiotically in a vertebrate gut without eliciting any potentially harmful host immune response. The mechanisms which govern the host-symbiont association patterns are not very well known. The microbial complexity of the insect and vertebrate guts is a major limiting factor in understanding the microbe-animal interactions in these models.

Photorhabdus is a gram negative enterobacterium which is found in nature only in the gut of the nematodes of the genus *Heterorhabditis* [4]. *Photorhabdus* are known to

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✉ Vishal S. Somvanshi
vssomvanshi@iari.res.in

[1] Division of Nematology, ICAR- Indian Agricultural Research Institute, Pusa Campus, New Delhi 110012, India

[2] ICAR-National Research Center on Plant Biotechnology, Pusa Campus, New Delhi 110012, India

[3] Department of Biotechnology and Biosciences, Banasthali Vidyapeeth, Jaipur, Rajasthan, India

produce an array of toxins and secondary metabolites [5, 6], and together with their nematode-hosts they are potent insect killers. The *Photorhabdus*–*Heterorhabditis* pair provides a tractable genetic model to study animal-microbes relationships [7]. These nematodes co-evolved with their bacterial symbionts for millions of years; and form very specific symbiotic relationships and mostly are not colonized by other strains and species of *Photorhabdus* [8]. Through many cross-colonization experiments performed in the laboratories, it has been established that *Photorhabdus* and *Heterorhabditis* symbiotic relationships are highly specific [8]. Since *Heterorhabditis* nematodes are used for the biological control of insects in agricultural crops, this bacteria-nematode specificity is an important factor for fermenter based commercial mass production of nematodes as the nematodes can multiply and develop only on their specific symbiont *Photorhabdus* strain [9, 10]. The genomes of the bacterial symbiont *P. luminescens* ssp. *laumondii* and its nematode-host *H. bacteriophora* TT01 have been sequenced [4, 11], and are available in public domain. Recently, the details of symbiont colonization sites in the nematode gut, and the developmental progression of this symbiotic relationship has been elucidated for *Photorhabdus*–*Heterorhabditis* pair [12]. Some of the bacterial genes and processes necessary for symbiotic colonization of the nematodes have also been identified [13–16].

Comparative genomics has been used to understand genomic diversification for niche expansion, for e.g. in *Lactobacillus* [17]. Similarly, genomics based approaches led to significant advancements in understanding the mechanisms involved in *Photorhabdus*–*Heterorhabditis* symbiosis, but our knowledge about the factors that govern host specificity *per-se* is rather limited. In the symbiosis between nematode *Steinernema carpocapsae* and its symbiont *Xenorhabdus nematophila*, it was found that *X. nematophila nilABC,* a single genetic locus encoding for membrane proteins was necessary and sufficient for determining initial colonization specificity [18]. The only study to determine genomic regions involved in host specificity in *Photorhabdus*–*Heterorhabditis* system by comparing gDNA of *P. luminescens* ssp *laumondii* TT01 with *P. temparata* XlNach strains in a microarray hybridization experiment identified 8 genomic regions possibly involved in host specificity [19]. However, lack of functional validation to support the findings in this study warranted further investigations.

The developments in the field of quantitative proteomics have revolutionized the field of biological research [20]. In case of insect parasitic nematodes and their symbiont bacteria, proteomics based global approaches were used in investigation of desiccation stress tolerance in entomopathogenic nematodes *Steinernema* [21], and for the analysis of phase variation process in the bacterium *Photorhabdus* [16]. We hypothesize that the host specificity may be caused by the differences in genetic constitution of the symbiont bacterial species, which might be reflected in their respective proteomes. Here, we used 2D-gel based approach to compare proteomes of two *Photorhabdus* species that do not colonize each other's nematode-host to identify the proteins that may govern host specificity between *Heterorhabditis* and *Photorhabdus*.

Materials and Methods

Strains and Culture Conditions

The nematode *H. indica* was isolated from Nagpur, Maharashtra, India (ITS accession No. HQ637414), and the nematode *H. bacteriophora* TTO1 was a gift from Dr. Byron Adams, BYU, USA to Dr. Sudershan Ganguly. Isolation of bacterial symbionts was done from the nematode infective juveniles (IJs). Freshly harvested IJs from White's trap were collected and surface sterilized by using 2 % commercial bleach and sterilized double distilled water. The surface sterilized IJs were manually crushed by tissue grinder, and one loop of this suspension was streaked on Petri-plates containing nutrient bromothymol blue agar (NBTA, in 1 litre water: 3 g beef extract, 5 g peptone, 0.025 g bromothymol blue and 0.04 g 2,3,5- triphenyl tetrazolium chloride, 15 g Agar, pH 7.2) and incubated at 28 °C for 48 h. Pure green colonies were isolated and confirmed as *Photorhabdus* by sequencing of 16s rDNA. The symbiont bacteria isolated from *H. indica* was identified as *P. luminescens* ssp. *akhurstii* (GenBank accession no. JX240394), and from *H. bacteriophora* as *P. luminescens* ssp. *laumondii*, and were used for isolation of total protein.

Isolation of Total Protein from Bacteria

The bacteria were cultured in Luria broth (LB) in an incubator shaker (28 °C, 48 h at 200 rpm). The cultures were centrifuged (6000 rpm, 8 °C) to harvest the cells. 10 mM sodium phosphate buffer (pH 6.0) was used to wash the bacterial cells and then the cells were mechanically lysed by grinding in Tris-HCl buffer (50 mM, pH 7.0) in a 1.5 ml tube. The cellular debris was removed by centrifuging the lysed cells and filtering the supernatant through 0.2 μm membrane (Millipore, India). The protein was quantitated using the Bradford method.

2-D Gel Electrophoresis and Staining

Cleaning of extracted proteins was done by 2-D Clean-Up kit (Amersham Biosciences, Belgium). The protein pellet

was rehydrated (rehydration buffer composition- 2 M Thiourea, 0.5 % Bioampholytes, 20 mM dithiothreitol, 7 M Urea). One hundred µg of purified protein was loaded on 11 cm IPG strips (GE Healthcare, USA) of pH 3–10 and 4–7. The first dimension separation of proteins was done on IEF cell (GE Healthcare, USA) at 200 V for 3 h. After this a gradient was applied for 2 h at 500 V, followed by final focusing at 8000 V for 2.5 h. The second dimension separation of proteins was done by using SDS-polyacrylamide gel (12.5 %), followed by silver staining. After the completion of electrophoresis run, the gels were incubated in a fixative solution (acetic acid-12 %, methanol-50 %), kept on rocking shaker at room temperature for 1 h, washed by 50 % ethanol followed by 30 % ethanol for 30 m, sensitized by 0.002 % sodium thiosulphate solution for 60 s, and then washed thrice for 20 s with distilled water. Silver staining of gel was done in 0.028 % formaldehyde and 12.0 mM $AgNO_3$ solution for 20 m, and the gel was washed thrice by distilled water prior to processing in the developing solution (sodium carbonate- 6 %, formaldehyde- 0.0185 % and sodium thiosulphate- 4 %). Lastly, the gel was scanned at 300 dpi resolution using a scanner (CanoScan 8400F, Canon, Germany) to acquire the image of gels for identification of host specific molecules. The unique proteins were identified, excised, and sent for MALDI-TOF/TOF analysis.

In–Gel Digestion and Protein Identification

The completely resolved unique spots were excised and dehydrated in a vacuum centrifuge prior to the enzymatic digestion. Rehydration of the gel slices was done in trypsin (10.0 ng/µl in ammonium bicarbonate (100 mM, pH 7.4). The tubes were incubated overnight at 37 °C to allow complete protein digestion; thereafter extracted peptides were vacuum dried. MALDI-TOF analysis was done by outsourcing to Sandor Life Sciences Pvt.Ltd., Hyderabad, India. In brief, 1.0 µl of the peptide sample (reconstituted) was added into 2 consecutive columns (C18-reversed phase chromatography, and C18 PepMap nano-analytical column (LC Packings, Germering, Germany)). MALDI-TOF with a positive ion mode nano-flow ESI Z-spray source was utilized for analyzing chromatographically separated peptides. The data was captured by MassLynx software (v 4.0), processed by ProteinLynx Global Server (v 2.2, Micromass, Manchester, U.K.) as PKL (peak list) at standard parameters. Peak lists of peptides were searched against MASCOT (http://www.matrixscience.com) and NCBI nr protein database at standard parameters. The protein was called as 'identified' when the threshold $P < 0.05$ was exceeded by peptide ion score for 2 or more peptides, and the molecular mass and pI values matched in the corresponding gel(s). The experiment was replicated thrice.

Results

Total protein profiles of two subspecies of *Photorhabdus* bacteria that do not colonize each other's nematode-host, i.e., *P. luminescens* ssp. *laumondii* (host- *H. bacteriophora*), and *P. luminescens* ssp. *akhurstii* (host- *H. indica*) were matched. Distinct differences in SDS-PAGE protein profiles were observed between 15–55 kDa molecular weight range suggesting proteomic divergence (Fig. 1). The differences in proteomes were further resolved using 2D-gel electrophoresis followed by sequencing of proteins unique to each bacterial subspecies. Isoelectric focusing of proteins at pH 3 to 10 revealed 4 unique spots in *P. luminescens* ssp. *akhurstii* and 8 in *P. luminescens* ssp. *laumondii,* respectively (Fig. 2a, b). Resolving the protein spots by expanding the pH range from 4 to 7 identified 11 and 16 additional unique spots in *P. luminescens* ssp. *akhurstii* and *P. luminescens* ssp. *laumondii,* respectively (Fig. 2c, d). In total, 39 unique protein spots were identified; 15 unique to *P. luminescens* ssp. *akhurstii* and 24 unique to *P. luminescens* ssp. *laumondii.*

These spots were identified by peptide mass mapping, followed by sequence search against UniProt (Table S1). The proteins unique to *P. luminescens* ssp. *akhurstii*, (Spot numbers 1–4, 13–23, Fig. 2, Table S1) were identified as O-acetylhomoserine amino carboxypropyl transferase; catalase (KatE); peptidase T (PepT); ABC transporter ATP-binding protein (AfuC); iron-containing alcohol dehydrogenase; ABC transporter-like protein (YhbG); hypothetical protein highly similar to probable transcription regulator AraC; hypothetical protein similar to conjugative transfer

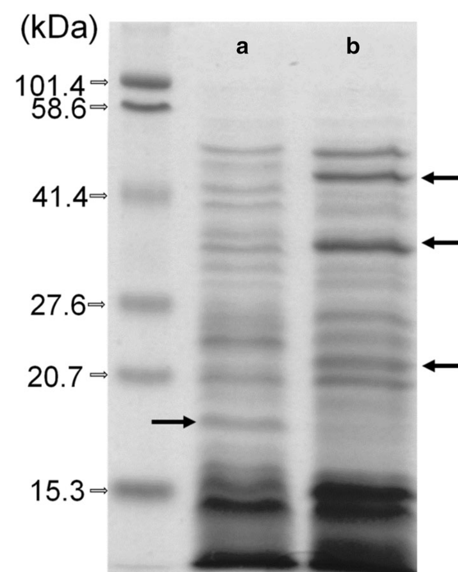

Fig. 1 Comparison of total protein profiles of **a** *P. luminescens* ssp. *akhurstii* and **b** *P. luminescens* ssp. *laumondii. Solid black arrows* indicate differentially expressed protein bands between the two *Photorhabdus* species. *Hollow arrows* indicate protein marker size

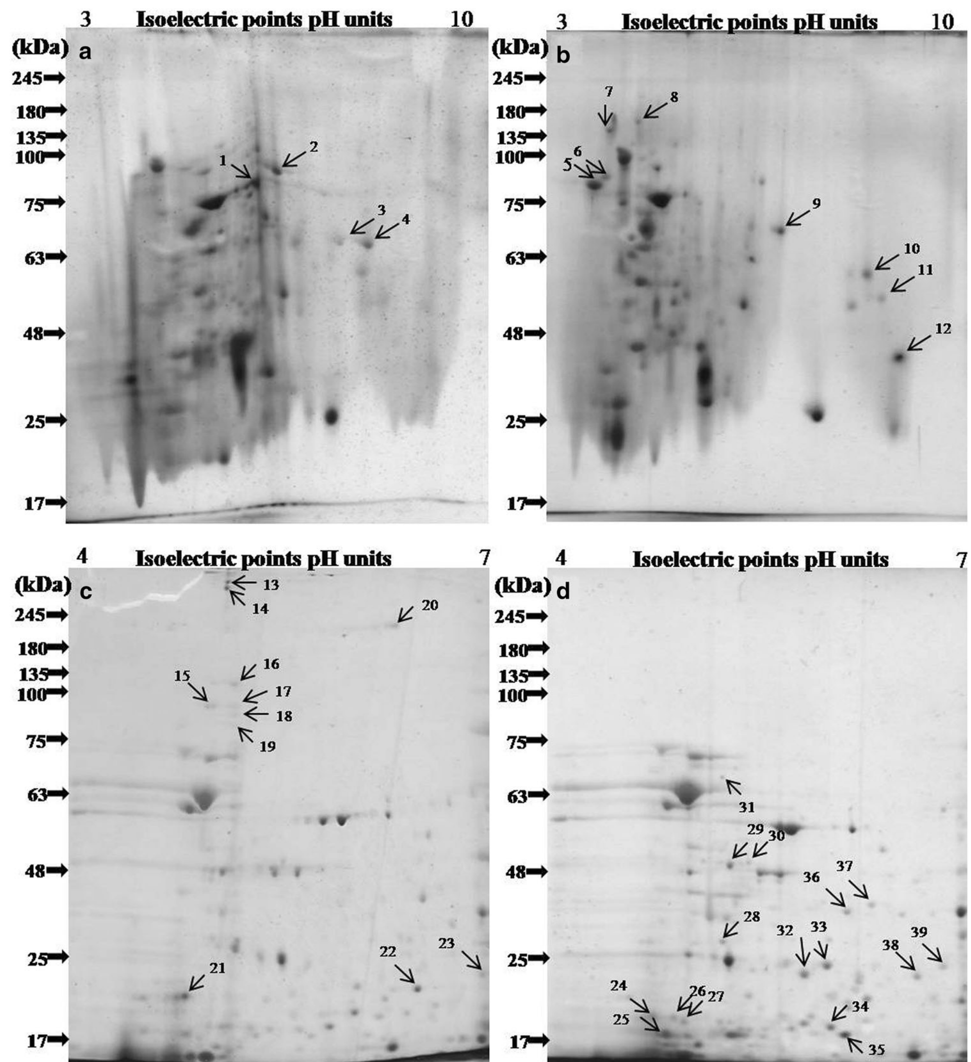

Fig. 2 2D-gel electrophoresis map of proteins at isoelectric point pH units 3–10 **a** *P. luminescens* ssp. *akhurstii* and **b** *P. luminescens* ssp. *laumondii* and isoelectric point pH units 4–7 **c** *P. luminescens* ssp. *akhurstii* and **d** *P. luminescens* ssp. *laumondii*. *Thin black arrows* indicate unique protein spots; *thick black arrows* indicate size of protein marker

ATPase of PFL_4706 family; DNA topoisomerase IV subunit A, ParC; hypothetical protein similar to YjeE of *Escherichia coli*; hypothetical protein similar to flavoprotein WrbA (Trp repressor binding protein); dihydrolipoamide dehydrogenase IpdA; radical SAM domain-containing protein PflA; pyridoxine 5′-phosphate synthase, PdxJ, and methylglyoxal synthase, MgsA.

Similarly, proteins those were unique to *P. luminescens* ssp. *laumondii* (spot numbers 5–12 and 24–39, Fig. 2, Table S1) comprised of 2 hypothetical proteins not similar to any know protein; molecular chaperone protein DnaK; elongation factor G FusA; outer membrane protein A (ompA); phosphate transporter subunit PstS; glutamate and aspartate transporter subunit GltI; hypothetical protein similar to non-ribosomal peptide synthetase modules and related proteins EntF; phosphate regulon sensor kinase PhoR; phospho carrier protein NPr, ptsO; hypothetical protein similar to YcjX of *E. coli*; TolB; L-isoaspartate carboxyl methyltransferase Pcm; enolase (2-phosphoglycerate dehydratase); sulfate transport system permease protein CysW; small subunit of the acetolactate synthase isozyme III (AHAS-III), IlvH; pilin FimA; malate dehydrogenase Mdh; 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase GpmA; triosephosphate isomerase TpiA; iron ABC transporter substrate-binding protein; amino-acid acetyltransferase (N-acetylglutamate synthase) ArgA; aromatic amino acid amino-transferase, AspC and molybdenum-binding transcriptional repressor ModE.

In summary, a set of 39 proteins was identified, 15 unique to *P. luminescens* ssp. *akhurstii* and 24 unique to *P. luminescens* ssp. *laumondii.*

Discussion

To identify proteins involved in conferring nematode-host specificity, we compared the total protein profiles of *P. luminescens* ssp. *laumondii* (nematode-host- *H.*

bacteriophora) and *P. luminescens* ssp. *akhurstii* (nematode-host- *H. indica*) by 2D-gel electrophoresis. Several proteins (Fig. 2, spots 4, 13, 14, 15, 16, 20, 24, 31) did not run at theoretical molecular weight (Table S1) resulting in an anomaly which is commonly known as 'gel shift'. Many membrane proteins are covalently bound to carbohydrates or lipid moiety hence it is expected that their molecular weight would be higher than theoretical molecular weight. However, several other factors are known to be responsible for this anomaly. The first reason could be the binding of proteins to SDS leading to formation of SDS-complexes and resulting in modification and oligomerization of proteins [22]. Other reasons could be post translational modifications like glycosylation, ubiquitination or phosphorylation etc., presence of acidic amino acids in the protein [23], incomplete unfolding of proteins, or presence of proline-rich regions which lend rigidity to the protein backbone. Lower observed molecular weight of some proteins could be due to truncation of proteins as some proteins carry protease sensitive motifs and may get cleaved off.

Some of the differences in the protein profile of the two *Photorhabdus* strains can definitely be attributed to the taxonomic differences between the compared strains. Few proteins identified as being unique in *P. luminescens* ssp. *akhurstii* are housekeeping proteins (for e.g. LpdA, ParC and KatE) and homologues are present in *P. luminescens* ssp. *laumondii* TTO1 based on genome sequence. Also, some proteins found as unique in *P. luminescens* ssp. *laumondii* TTO1 are important and conserved proteins (i.e. DnaK, Mdh), and are likely to be present in *P. luminescens* ssp. *akhurstii*. Some other proteins identified in our study are required for secondary metabolite biosynthesis, for e.g. pyruvate formate-lyase PflA, flavo protein wrbA, Pyridoxine 5′-phosphate synthase PdxJ. We found these proteins as unique to either of the bacterial strains in this study. It is unlikely that these proteins are directly involved in determination of host specificity. However, these genes might regulate bacterial secondary metabolism in post-exponential growth phase and production of symbiosis factors (for e.g. bioluminescence, an anthraquinone pigment (AQ) and an antibiotic 3-5-dihydroxy-4-isopropylstilbene), and might affect host specificity and symbiosis indirectly. For e.g., Plu4547 (*mdh*) was previously identified to be involved in symbiosis between *Photorhabdus* and *Heterorhabditis* [14]. *mdh* encodes for malate dehydrogenase which is a key tricarboxylic acid (TCA) cycle enzyme. It was observed that nematode growth and development was not supported by an *mdh* mutant, establishing the role for secondary metabolism in bacteria-nematode symbiotic interactions [14]. Similarly, an alarmone (p)ppGpp is needed for the symbiont bacteria *P. luminescens* to sustain nematode development and growth through its effect on secondary metabolism. Therefore, without an in-depth investigation on role of each one of these housekeeping, essential and biosynthetic pathway proteins, it would be premature to speculate on their role in host specificity and symbiosis.

A subset of the identified proteins could be directly involved in modulating nematode-bacterium host specificity. Three proteins, i.e. O-acetylhomoserine amino carboxypropyl transferase (Plu3517); hypothetical protein, similar to non-ribosomal peptide synthetase modules and related proteins EntF (Plu3130); and P pilus assembly protein, pilin FimA (Plu0418) are present in *P. luminescens* ssp. *laumondii* TTO1, which is the bacterial symbiont of *H. bacteriophora,* but not in *P. temperata* ssp. *temperata* XlNach (host nematode-*H. megidis*). These three genes are present on three of the *Photorhabdus* genomic islands (GI) [4, 19]. The EntF protein is required for synthesis of a high affinity siderophore enterobactin [24]. It is a widely known fact that siderophores are involved in pathogenicity and host specificity of plant and animal pathogenic bacteria. A unique siderophore molecule might help the bacterial symbiont in colonizing its nematode-host. However, it remains to be seen if the structure of the same siderophore molecule varies between different *Photorhabdus* strains and species, and if this variability could lead to host specificity. Similarly, the pilin protein FimA is important in context of host specificity as fimbriae are bacterial adhesive organelle and facilitate host-nematode recognition and adhesion of the symbiont bacteria to a yet unknown receptor in the nematode intestine. It is possible that during the course of evolution, the pilins have co-evolved along with this nematode receptor leading to a high degree of host specificity recognition. Previously, a novel *mad* fimbrial locus (Plu0261-Plu0270) was found to be involved in symbiosis between the nematode and the bacteria, which supports this hypothesis [15, 25].

Discovering Plu1775 (outer membrane protein A) as unique in one of the strains is a significant finding. In a previous study to determine the factors responsible for host specificity in the bacterium *X. nematophila,* it was discovered that *nilABC* genes suggested to encode for various components of bacterial membrane proteins that were absent in eight other *Xenorhabdus* species but were present only in *X. nematophila*, thus underlining their role in host colonization and host specificity. Transfer of *X. nematophila nilABC* genes into two species of *Xenorhabdus* that did not colonize *S. carpocapsae* (*X. poinarii* and *X. bovienii*) enabled these species to symbiotically colonize *S. carposapsae* [18]. Similarly, in the Gram-negative bacteria, protein OmpA constitutes the outer membranes [26]. OmpA has many functions, including adherence to host tissues in several pathogenic bacteria (summarized in [26]). Difference in OmpA hypervariable domains has been

linked to host specificity between cattle and sheep isolates of *Mannheimia* (*Pasteurella*) *haemolytica*, *M. glucosida*, and *P. trehalosi* [26]. Therefore, it is possible that *Photorhabdus* OmpA might be important in establishing symbiosis and host specificity with their nematode-hosts.

In addition, we identified components of 5 different ABC transporters-*afuC* (Plu0810), *yhbG* (Plu4040), *pstS* (Plu0214) from *P. luminescens* ssp. *akhurstii*, *gltI* (Plu1307) and Plu2853 from *P. luminescens* ssp. *laumondii.* A group of ABC transporters, *lsr* locus, was identified as one of the regions common and specific to *Photorhabdus* bacteria that formed symbiotic relationships with *H. bacteriophora* in a previous study [19]. The *lsr* locus encodes for proteins suggested to be involved in quorum sensing [19]. Therefore, there is possibility that other ABC transporter systems are involved in symbiosis through similar mechanisms. For e.g., in a phosphate ABC transporter, PstS is a phosphate-binding component also suggested to be involved in two-component signal transduction [27] whereas role for *yhbG* is not clear. Function of other ABC transporters identified in this study, i.e., ferric (afuC, Plu2853), glutamate and aspartate (*GltI*) transport are relatively well defined. Signaling is extremely important in determining the outcomes of host-bacterium interactions.

Two unique putative transcriptional regulators- Plu2244 and Plu1964 from *P. luminescens* ssp. *akhurstii*, and one (Plu1474) from *P. luminescens* ssp. *laumondii* TTO1 were discovered in this study. Plu2244 is a hypothetical protein similar to *E. coli* transcription factor AraC. AraC is a transcriptional regulator of arabinose catabolism and transport genes and operons [28]. Plu1964 is similar to transcriptional repressor *wrbA* which has role in stress responses [29]. Plu1474 encodes for a protein similar to a molybdate-dependent transcriptional regulator ModE, which in *E. coli* acts as a molybdate concentration sensor, and regulates transcription of operons involved in molybdenum uptake and utilization [30]. Other interesting genes identified in our study were five novel hypothetical proteins, Plu2059, Plu2582, Plu4585, Plu2060, and Plu1085. It has been found in more than one case that a single gene could regulate the bacterial host range, and thus specificity. The transcriptional regulators and hypothetical proteins are always an interesting candidate for the functional validation because they could be involved in a biological phenomenon in a number of unpredictable ways.

In summary, we identified a pool of 39 bacterial proteins that were different between *P. luminescens ssp. akhurstii* and *P. luminescens* ssp. *laumondii* TTO1. It is likely that a subset of these proteins, for e.g. pilin FimA, OmpA, might actually be involved in regulating host specificity and symbiosis with their nematode-hosts, either directly, or indirectly via their effect on secondary metabolism. These proteins present interesting targets for functional genetic validation to confirm their role in governing nematode-bacterium host specificity and symbiosis.

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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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