Genetic and Proteomic Characterization of rpoB Mutations and Their Effect on Nematicidal Activity in Photorhabdus luminescens LN2

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Abstract

Rifampin resistant (RifR) mutants of the insect pathogenic bacterium Photorhabdus luminescens LN2 from entomopathogenic nematode Heterorhabditis indica LN2 were genetically and proteomically characterized. The RifR mutants showed typical phase one characters of Photorhabdus bacteria, and insecticidal activity against Galleria mellonella larvae, but surprisingly influenced their nematicidal activity against axenic infective juveniles (IJs) of H. bacteriophora H06, an incompatible nematode host. 13 out of 34 RifR mutants lost their nematicidal activity against H06 IJs but supported the reproduction of H06 nematodes. 7 nematicidal-producing and 7 non-nematicidal-producing RifR mutants were respectively selected for rpoB sequence analysis. rpoB mutations were found in all 14 RifR mutants. The rpoB (P564L) mutation was found in all 7 mutants which produced nematicidal activity against H06 nematodes, but not in the mutants which supported H06 nematode production. Allelic exchange assays confirmed that the Rif-resistance and the impact on nematicidal activity of LN2 bacteria were conferred by rpoB mutation(s). The non-nematicidal-producing RifR mutant was unable to colonize in the intestines of H06 IJs, but able to colonize in the intestines of its indigenous LN2 IJs. Proteomic analysis revealed different protein expression between wild-type strain and RifR mutants, or between nematicidal-producing and non nematicidal-producing mutants. At least 7 putative proteins including DsbA, HlpA, RhlE, RplC, NamB (a protein from T3SS), and 2 hypothetical proteins (similar to unknown protein YgdH and Yggc of Escherichia coli respectively) were probably involved in the nematicidal activity of LN2 bacteria against H06 nematodes. This hypothesis was further confirmed by creating insertion-deletion mutants of three selected corresponding genes (the downregulated rhlE and namB, and upregulated dsbA). These results indicate that the rpoB mutations greatly influence the symbiotic association between the symbionts and their entomopathogenic nematode hosts.

Introduction

Rifampin (Rif), first introduced in 1972 as an antitubercular drug, was initially extremely effective against Mycobacterium tuberculosis, and other bacteria [1–2]. With its widespread and extended use, the number of bacterial isolates resistant to Rif has increased. Most Rif-resistance mutations in M. tuberculosis as well as in Escherichia coli and Staphylococcus aureus were conferred by a set of restrictive mutations in the rpoB gene, which encoded the β-subunit of RNA polymerase (RNAP) in bacteria [3–4]. DNA-dependent RNAP, which contains an essential catalytic core enzyme (α2ββ′α′) and one of the sigma (σ) factors, is the central enzyme for expression of genomic information in all organisms. Rif inhibits transcription initiation by blocking the σ70 subunit of bacterial RNAP [5–6]. E. coli rpoB mutations that suppress the auxotrophy due to lack of stringent response were demonstrated to affect the transcription of stringently controlled genes by destabilizing the RNAP-stable RNA promoter complex [7]. The Rif resistant (RifR) M. tuberculosis mutations of the rpoB gene were found in nearly 95% of clinical isolates [4]. Most of the mutations were located from nucleotides 1276 to 1356 (codon 432–458 in M. tuberculosis rpoB gene and codon 307–333 in E. coli rpoB gene). An 81 bp core region was called the Rif resistance determining region (RRDR) of rpoB [8–10]. However, a significant number of RifR mycobacteria with no mutations in the rpoB gene have been isolated from different clinical samples [11–14]. A 191A/C mutation in the Rv2629 gene was reported to be significantly associated with Rif-resistance in M. tuberculosis [15]. Recently, it was reported that the K1 uptake regulator TrkA played an important role in intrinsic and acquired antibiotic resistance in mycobacteria [16]. Besides Rif-resistance, the rpoB mutation (Δ621E) conferred dual heteroresistance to daptomycin and vancomycin in Staphylococcus aureus [17], but most rpoB mutations were involved in reduced vancomycin susceptibility [18]. It suggested that different rpoB mutations may have different effect on bacteria.

Photorhabdus and Xenorhabdus bacteria belonging to the Enterobacteriaceae are symbiotically associated respectively with entomopathogenic Heterorhabditis and Steinernema nematodes, which are used as a commercial bioinsecticide for many economically important insect pests [19]. The association between the
Characterization of Mutants and Wild-type Bacterial Strain

Colonial Characterization of the Mutants and Wild-type Bacterial Strain

Colonization was determined on LB1 agar, NBTA, and MacConkey agar plates. pH-sensitive pigment production in LB1 was determined by addition of 1 M NaOH or 1 M HCl. Tests for the production of antibiotic substances were conducted as previously described [48], using Bacillus subtilis as test organism, and scored positive when a growth inhibition zone of >3 mm was measured around the P. luminescens colonies at 96 h after inoculation of the overlay culture. Bioluminescence was observed by dark-adapted eyes in a dark room. Cell morphology was observed microscopically. Catalase activity was tested by introducing 0.1 ml 3% hydrogen peroxide into the bacterial cultures and observing the release of oxygen. For all assays, both wide-type and mutant colonies were characterized on the same agar plate ad positive and negative controls. At least three plates for each medium were established.
Plasmid DNA preparation, extraction of genomic DNA, restriction enzyme digestions, and ligations were carried out as previously described [49]. Restriction enzymes (Promega, USA) and T4 ligase (Novagen, Germany) were used according to the manufacturer’s instructions. Plasmids were extracted from *E. coli* with QIAprep Spin Miniprep kit (Qiagen, Netherlands). When required, DNA fragments were extracted and purified from agarose gels using E.Z.N.A.™ Gel Extraction kit (Omega, USA). The genomic DNA was isolated from *P. luminescens* bacteria using E.Z.N.A.™ Bacterial DNA Kit (Omega).

**Table 1.** Bacterial strains and plasmids used in this study.

| Strain/plasmid | Description, relevant characteristics | Reference or source |
|----------------|--------------------------------------|---------------------|
| **Photorhabdus luminescens** strains | | |
| LN2/LN2-W | Wild-type isolate from host nematode H. indica LN2 | Ralf-Udo Ehlers |
| LN2-R1—LN2-R34 | Spontaneous RifR mutant of wild-type strain LN2; RifR | This study |
| LN2-A | Spontaneous AmpR mutant of wild-type strain LN2; AmpR | This study |
| LN2-M1 | Tn5 insertion mutant of LN2; AmpR, KmR | [41] |
| H06 | Wild-type isolate from host nematode H. bacteriophora H06 | Laboratory stock |
| HNA | Wild-type isolate from host nematode H. megidis HNA | Dr Wim Wouts |
| LN2-WΔrpoB-LR31 | LN2-W containing the mutant rpoB allele from LN2-R31 | This study |
| LN2-R31ΔrpoB-LW | LN2-R31 containing the wild-type rpoB allele from LN2-W | This study |
| LN2ΔrhlE | LN2-A rhlE::Cm | This study |
| LN2ΔnamB | LN2-A namB::Cm | This study |
| LN2ΔdsbA | LN2-A dsbA::Cm | This study |
| **Escherichia coli** strains | | |
| DH5α | Host of plasmids | TaKaRa |
| S17-1 (ispI) | *E. coli* lysogenized with ispI, replication of ori R6K | [51] |
| TOP10 | Cloning strain | Invitrogen |
| **Plasmids** | | |
| pMini-Tns | oriT, oriR6K, delivery plasmid for mini-Tns; KmR | [52] |
| pCR4-TOPO | Cloning vector; AmpR, KmR | Invitrogen |
| pMD-18T | Cloning vector; AmpR | TaKaRa |
| pMD-19T | Cloning vector; AmpR | TaKaRa |
| pUC19-egfp | pUC19 carrying egfp; AmpR | Laboratory stock |
| pMD-egfp | pMD-18T carrying ribosome binding site and egfp gene; AmpR | This study |
| pMD-lac-egfp | pMD-19T carrying ribosome binding site, lacZ promoter and egfp gene; AmpR | This study |
| pMini-lac-egfp | pMini-Tns carrying a NotI fragment containing ribosome binding site, lacZ promoter and egfp gene; KmR | This study |
| pPHU281 | lacZ' mob(RP4), TcR derivative of pUC18 with oriT | [50] |
| pPHU281ΔrpoB-LW | pPHU281 carrying a BamHI-PstI fragment containing rpoB gene from wild-type strain of *P. luminescens* LN2 | This study |
| pPHU281ΔrpoB-LR31 | pPHU281 carrying a BamHI-PstI fragment containing rpoB gene from RifR mutant of *P. luminescens* LN2 | This study |
| pNG101 | R6K Ori, sacB, SmR | [56] |
| pNG101ΔrhlE::Cm | pNG101 carrying a rhlE::Cm fragment, SmR, KmR | This study |
| pNG101ΔnamB::Cm | pNG101 carrying a namB::Cm fragment, SmR, KmR | This study |
| pNG101ΔdsbA::Cm | pNG101 carrying a dsbA::Cm fragment, SmR, KmR | This study |

DNA Manipulation

Plasmid DNA preparation, extraction of genomic DNA, restriction enzyme digestions, and ligations were carried out as previously described [49]. Restriction enzymes (Promega, USA) and T4 ligase (Novagen, Germany) were used according to the manufacturer’s instructions. Plasmids were extracted from *E. coli* with QIAprep Spin Miniprep kit (Qiagen, Netherlands). When required, DNA fragments were extracted and purified from agarose gels using E.Z.N.A.™ Gel Extractio kit (Omega, USA). The genomic DNA was isolated from *P. luminescens* bacteria using E.Z.N.A.™ Bacterial DNA Kit (Omega).

**Mutation Analysis of the rpoB Gene from Different Strains**

To examine the rpoB sequence from the RifR mutants and wild-type strain (LN2-W), together with a spontaneous AmpR mutant LN2-A and the namA mutant LN2-M1 [41], the gene was amplified from the genomic DNA of different strains, by PCR with PfuUltra™ II Fusion HS DNA Polymerase (Stratagene, Germany), using the primers rpoB-BamHI-F (5’-GCTGGATC-CATGGTTTACTCCTATACCGAG-3’) and rpoB-PstI-R (5’-GCATGAGTTATTCGTCTTCCCAGTGCGATG-3’). The amplified gene was cloned into pCR4-TOPO vector (Invitrogen, USA), and transformed into *E. coli* TOP10 (Invitrogen). DNA sequencing was performed by Invitrogen Trading (Shanghai) Co. Ltd. All strains were sequenced at least twice. The sequence data of the rpoB gene were assembled and analyzed with DNAstar and CLUSTAL W program. The rpoB sequence data from wild-type strain of *P. luminescens* LN2 has been submitted to the GenBank database under accession number (JN177303).

**Allelic Exchange Mutagenesis of the rpoB Gene**

The plasmids of pCR4-TOPO-rpoB-LW and pCR4-TOPO-rpoB-LR31 containing corresponding rpoB genes from LN2-W and LN2-R31, were digested with BamHI (GGATCC) and PstI (GCATGAGTTATTCGTCTTCCCAGTGCGATG).
(CTGCAG), respectively. The resulting rpob fragments were purified and ligated into the suicide vector pPHU281 [50] digested with BamHI and PsI to yield plasmids pPHU281-rpob-LW and pPHU281-rpob-LR31. The resulting plasmids were transferred into E. coli S17-1 (lpir) [51]. Strains LN2-WΔrpob-LR31 (LN2-W containing the mutant rpob allele from LN2-R31) and LN2-R31Δrpob-LW (LN2-R31 containing the wild type rpob allele from LN2-W) were created by allelic exchange with pPHU281-rpob-LR31 and pPHU281-rpob-LW, respectively, using biparental mating method. RifR.AmpR.TcS exconjugants of LN2-WΔrpob-LR31 and RifS.AmpR.TcS exconjugants of LN2-R31Δrpob-LW were selected on LB1 agar plates with appropriate antibiotics. The exconjugants had undergone allelic exchange and lost the wild-type or mutated copy of rpob and the plasmid vehicle. The mutants were verified for the presence of the appropriate rpob allele by sequencing rpob gene as described above. The resulting confirmed allelic exchange mutants were determined for the nematicidal activities against the IJs of H06 as described above.

### Table 2. rpob mutations of the RifR mutants and their effect on H06 nematode growth.

| Bacterial mutants | Nucleotide change | Amino acid change | Effect on the growth of H06 nematodes |
|-------------------|------------------|------------------|--------------------------------------|
| LN2-R2            | G436T            | V146F            | +                                    |
| LN2-R6            |                  |                  |                                      |
| LN2-R12           | C938A            | A313D            | +                                    |
|                   | C1585T           | R529C            |                                      |
| LN2-R28           | C1535T           | S512F            |                                      |
| LN2-R33           | C1537A           | Q513K            | +                                    |
| LN2-R3            | C1691T           | P564L            |                                      |
| LN2-R7            |                  |                  |                                      |
| LN2-R11           |                  |                  |                                      |
| LN2-R16           |                  |                  |                                      |
| LN2-R25           |                  |                  |                                      |
| LN2-R5            | C521T            | A174V            | -                                    |
|                   | C1691T           | P564L            |                                      |
| LN2-R8            | C826T            | Q276*            | -                                    |
|                   | C1691T           | P564L            |                                      |
| LN2-A             | No change        | No change        |                                      |
| LN2-M1            | No change        | No change        |                                      |

* Nematode production;  
+ No nematode production and nematode died after 7 days;  
2 No nematode production and nematode died after 7 days;  
*stop code.  
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Insecticidal Injection Assays

To check the insecticidal activity of the RifR mutants, the wild-type strain and RifR mutants of P. luminescens LN2 were grown overnight in LB1 broth without antibiotics, subcultured into fresh LB1 broth with 1% of bacterial culture, and incubated at 25°C for 24 h prior to injection. These cultures were washed and diluted to concentrations of 10, 100, 1000 CFU/µL in sterile phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO4, 2 mM KH2PO4, pH 7.4). Last instar larvae of greater wax moth Galleria mellonella were incubated on ice for approximately 3 min. 10 µL of the diluted cultures or sterile PBS were injected into the first proleg of each of 10 insect larvae using a 30-gauge syringe (Hamilton, Reno, NV). Three replicates with 10 insect larvae per replicate were established. Insects were monitored every 6 h for 120 h post injection. Dead insects were observed to confirm the presence of red color and bioluminescence.

Colonizations of H06 IJs by the GFP-labelled Mutants

To observe the colonization of IJ nematodes by the RifR mutant bacteria, the RifR mutant LN2-R31 positive for the growth of H06 nematodes was labeled with GFP by transposon mutagenesis of a pMini-Tn5 [52] containing an expressed egfp gene (pMini-lac-egfp). The pMini-lac-egfp was constructed as follows. A fragment containing an egfp gene and ribosome binding site (GAAGGTTTAGACATGGGCAAAGGAGA) was obtained from pUC19-egfp with primers egfp-SD2 (59- GAAGGTTTAGACATGGGCAAAGGAGA-39) and egfp-rev (59- TAACGCGGCCGCTTATTTGTATAGTTCATC-39) (NotI). The amplified 750 bp PCR product was cloned into the pMD-18T vector (TaKaRa, Japan) and transformed into E. coli DH5a. Green clone on LB2 plates with ampicillin was selected to extract the plasmid pMD-egfp. A NotI-NotI fragment containing the lac promoter and egfp gene from pMD-egfp was cloned into pMD-19T Simple vector (TaKaRa) to generate pMD-lac-egfp, with primers lac-F (59- ACGCGGCCGCTTATTTGTATAGTTCATC-39) (NotI) and egfp-rev (NotI). After transformed into E. coli DH5a, clones (AmpR) expressing GFP were detected using epifluorescence microscope (Nikon Eclipse 80i). To construct a transposon delivery vector pMini-lac-egfp, a NotI-NotI fragment containing the lac promoter and egfp gene from pMD-lac-egfp was cloned into the pMD-18T vector (TaKaRa). The automated 750 bp PCR product was cloned into the pMini-Tn5 vector (TaKaRa, Japan) and transformed into E. coli S17-1 (lpir). Clones

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homogenized nematodes on LB1 agar plates. was determined by plating dilutions of surface-sterilized and presence of the GFP-labeled bacteria retained in the IJs intestines. 

To check the colonization of H06 IJs by the RifR mutant LN2-R31, the nematodes were cultured on sponge medium [30], inoculated with GFP-labeled LN2-R31 as described above, respectively. The IJs were extracted from the sponge and observed for GFP-labeled bacteria. The IJs were also homogenized with a sterile glass homogenizer after surface sterilization with 0.1% merthiolate and 5-time rinse with sterile distilled water. The presence of the GFP-labeled bacteria retained in the IJs intestines was determined by plating dilutions of surface-sterilized and homogenized nematodes on LB1 agar plates.

2-DE Analysis and Protein Identification by MALDI-TOF-MS

The 48 h old bacterial cells of the wild-type strain and RifR mutants (one negative mutant LN2-R16 and three positive mutants LN2-R2, LN2-R31 and LN2-R33, for H06 growth) grown on LB1 plates at 25°C were harvested and washed three times with cold PBS by centrifugation (6000 g, 10 min, 4°C). The cell pellets were resuspended in lysis buffer (0.1 M urea, 0.2% w/v Bio-Lyte 3/10 Ampholyte (Bio-Rad, USA), 4% CHAPS, 65 mM DTT) containing Protease Inhibitor Cocktail (Calbiochem, Germany) and Benzonase (Novagen, Germany) and disrupted by liquid nitrogen freezing-thawing three times. Cell debris was removed by centrifugation (20000 g, 60 min, 4°C). The supernatant (total cell protein) was divided into aliquots and stored at -80°C until use. Protein concentrations were determined by the Bradford method using Modified Bradford Protein Assay Kit (Sangon, China).

The 2-DE was performed according to the methods described previously [53] and the manufacturer’s instruction. The first dimension (isoelectric focusing) was conducted using the IGPPhor IEF system (Bio-Rad) at 20°C. For analytical gels, 350 μg protein was solubilized in 400 μL rehydration solution (8M urea, 0.2% w/v Bio-Lyte 3/10 Ampholyte, 4% CHAPS, 65 mM DTT, 0.01% w/v bromophenol blue), and loaded onto a 17 cm pH 3–10 NL IPG strip (Bio-Rad). Focusing was performed for 13 h at 50V, 1 h at 500 V, 1 h at 1000 V, and 5 h and 30 min at 8000 V (total = 45 kVh). The IPG strips were equilibrated as previously described [53]. The second dimension was performed with 12% (w/v) SDS-polyacrylamide gels using the Protean II xi 2D protein separation system (Bio-Rad). The IPG strips were equilibrated as previously described. The supernatant (total cell protein) was divided into aliquots and stored at -80°C until use. Protein concentrations were determined by the Bradford method using Modified Bradford Protein Assay Kit (Sangon, China).

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Spots of interest in gels staining with silver nitrate were cut out, washed, reduced, S-alkylated with iodoacetamide and in-gel digested at 37°C overnight with sequencing grade porcine trypsin (Promega, USA). After extraction in extractant of 50% ACN (Fisher) and 2.5% TFA (Sigma), peptide mixtures were analyzed using a saturated solution of 5 mg/mL 2-cyano-4-hydroxycinnamic acid (CHCA, Sigma) in ACN containing 0.1% TFA (Sigma) (50/50 v/v) using a 4800 Proteomics Analyzer equipped with matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Applied Biosystems, Framingham, MA, USA). For MS calibration, the trypsin autolysis peptides were used as internal calibrants. Monoisotopic peak masses were automatically determined within the mass range of 800–4000 Da, with a minimum S/N of 50. Five of the most intense ion signals were selected as precursors for MS/MS acquisition. Combined MS and MS/MS queries were performed with the Mascot search engine (V2.1, Matrix Science, UK) embedded in GPS-Explorer Software (V3.6, Applied Biosystems), using the P. luminescens database (Gene DB). Mascot protein scores (based on combined MS and MS/MS spectra) of greater than 61 were considered statistically significant (p < 0.05). The individual MS/MS spectrum with statistically significant (confidence interval >95%) best ion score (based on MS/MS spectra) were also accepted.

Insertion-deletion Mutations of the Corresponding Genes from Differentially Expressed Proteins

Compared with the nematicidal-producing mutant LN2-R16 and wild type strain, four proteins including RplC, RhlE, NamB (a putative transport and binding protein from type III secretion system), and a hypothetical protein similar to unknown protein YgdH of E. coli were downregulated in the non-nematicidal-producing mutants LN2-R2, LN2-R31 and LN2-R33 (Table 3–4, Figure S1, S2, S3, S4, S5). At least these 7 putative proteins were probably involved in the nematicidal activity of LN2 bacteria against H06 nematodes.

To confirm these results, the downregulated rhlE and namB, and upregulated dsbA, HlpA, and a hypothetical protein highly similar to unknown protein YgdH of E. coli were upregulated in the non-nematicidal-producing mutants LN2-R2, LN2-R31 and LN2-R33 (Table 3–4, Figure S1, S2, S3, S4, S5). At least these 7 putative proteins were probably involved in the nematicidal activity of LN2 bacteria against H06 nematodes.

Results

Isolation and Characterization of the RifR Mutants of P. luminescens LN2

Several hundreds of the RifR mutants of P. luminescens LN2 were isolated and 34 mutants were randomly selected for further study. The wild type strain and the selected mutants showed the typical
| Spot No. | Protein Name/plu | Organism | Gene | Function | Protein PI | Protein MW | Ratio |
|---------|-----------------|----------|------|----------|------------|------------|-------|
| Ribosomal protein | | | | | | | |
| 6 | 30S ribosomal protein S8 | *P. luminescens* TT01 | *rpsH* | Binds directly to 16S rRNA central domain where it helps coordinate assembly of the platform of the 30S subunit | 9.35 | 14205.6 | 1 0.7 0.8 0.27 1.03 |
| 15 | 50S ribosomal protein L17, plu4701 | *P. luminescens* TT01 | *rplQ* | A component of the macrolide binding site in the peptidyl transferase center | 11.04 | 14708.8 | 1 0.5 1.27 0.69 1.34 |
| 26 | 50S ribosomal protein L3, plu4726 | *P. luminescens* TT01 | *rplC* | Binds directly near the 3’ end of the 23S rRNA, where it nucleates assembly of the 50S subunit; essential for peptidyltransferase activity; mutations in this gene confer resistance to tiamulin | 9.87 | 22328.9 | 1 0 0.41 0 0 |
| 44 | 50S ribosomal protein L9, plu4570 | *P. luminescens* TT01 | *rplI* | In *E. coli* this protein is wrapped around the base of the L1 stalk | 6.13 | 15872.5 | 1 2.38 2.23 0.92 1.37 |
| 45 | 50S ribosomal protein, L9, plu4570 | *P. luminescens* TT01 | *rplI* | In *E. coli* this protein is wrapped around the base of the L1 stalk | 6.13 | 15872.5 | 1 1.82 3.29 0.51 1.23 |
| Adaptations conditions | | | | | | | |
| 7 | Hypothetical protein, plu2032 | *P. luminescens* TT01 | Similar to Unknown protein YbdQ of *E. coli*; Similar to universal stress protein (pfam00582: Usp) | 6.19 | 15907.4 | 1 3.79 2.9 1.46 4.83 |
| 9 | Hypothetical protein, plu2030 | *P. luminescens* TT01 | Similar to Unknown protein YbdQ of *E. coli*; Similar to universal stress protein (pfam00582: Usp) | 5.76 | 15283.2 | 1 1.71 1.21 0.2 0.45 |
| 48 | Alkyl hydroperoxide reductase, small subunit (antioxidant), plu3907 | *P. luminescens* TT01 | *ahpC* | Alkyl hydroperoxide reductase, small subunit (antioxidant) | 5.92 | 22259.3 | 1 0.8 0.82 0.9 0.47 |
| Secondary metabolites | | | | | | | |
| 12 | Crystalline inclusion protein CipB | *P. luminescens* TT01 | *cipB* | Unknown, similar to crystalline inclusion protein type II | 6.08 | 11281.6 | 1 0.12 0 0.51 0 |
| 25 | Crystalline inclusion protein CipA, plu1576 | *P. luminescens* TT01 | *cipA* | Crystalline inclusion protein CipA | 6.06 | 11710.9 | 1 0 0.08 0.74 0 |
| 32 | Unknown | *P. luminescens* | Similar to hemolysin from *Fusobacterium nucleatum* clinical isolate found in GenBank, Accession Number AF525507 | 5.61 | 39995.2 | 1 0.9 1.7 0.44 0.8 |
| 62 | Hypothetical protein, plu4211 | *P. luminescens* TT01 | Highly similar to Hcp protein | 6.29 | 18482.5 | 1 11.43 14.91 19.98 3.24 |
| Spot No. | Protein Name/plu | Organism | Gene | Function | Protein PI | Protein MW | W/W | R2/W | R16/W | R31/W | R33/W |
|----------|-----------------|----------|------|----------|------------|------------|------|------|-------|-------|-------|
| **Metabolism of amino acids and related molecules** | | | | | | | | | | | | |
| 30 | Ethanolamine ammonia-lyase small subunit, plu2971 | P. luminescens TT01 | euC | Catalyzes the formation of acetaldehyde from ethanolamine | 6.37 | 31188.4 | 1 | 4.17 | 0.5 | 1.1 | 1.2 |
| 36 | Tryptophan synthase subunit beta, plu2466 | P. luminescens TT01 | trpB | Catalyzes the formation of L-tryptophan from L-serine and 1-indol-3-ylglycerol 3-phosphate | 6.2 | 43098 | 1 | 1.22 | 0.25 | 0.41 | 0.58 |
| 46 | Serine/arginine repetitive matrix protein 2 | | Srm2 | Converts cis-3-(3-carboxyethyl)-3,5-cyclohexadiene-1,2-diol (PP-dihydrodiol) into 3-(2,3-dihydroxy/phenyl) propionate | 12.02 | 294500.8 | 1 | 0.72 | 1.7 | 0.89 | 0.9 |
| 50 | 2,3-dihydroxy-2,3-dihydrophenylpropionate dehydrogenase, plu2207 | P. luminescens TT01 | hcaB | Convert 2,3-dihydroxy-2,3-dihydrophenylpropionate dehydrogenase | 5.43 | 29519.1 | 1 | 0.37 | 0.3 | 0.49 | 0.9 |
| 51 | Hypothetical protein, plu4676 | P. luminescens TT01 | | Similar to 3-oxoacyl-[acyl-carrier-protein] synthase II (beta-ketoacyl-ACP synthase II) (KAS II) | 5.58 | 45407.3 | 1 | 3.29 | 3.0 | 0.01 | 0.01 |
| 52 | Serine hydroxymethyltransferase, plu2931 | P. luminescens TT01 | glyA | Catalyzes the reaction of glycine with 5,10-methylenetetrahydrofolate to form L-serine and tetrahydrofolate | 5.92 | 45229.8 | 1 | 3.74 | 4.55 | 2.93 | 5.6 |
| 54 | Hypothetical protein, plu2040 | P. luminescens TT01 | ureG | Urease accessory protein | 5.04 | 22865.2 | 1 | 4.2 | 2.98 | 5.17 | 6.66 |
| 55 | 3-oxoacyl-(acyl carrier protein) synthase III, plu2835 | P. luminescens TT01 | fabH | FabH; beta-ketoacyl-acyl carrier protein synthase III; converts 3-oxoacyl-ACP to initiate cycles of fatty acid elongation; differs from 3-oxoacyl-(acyl carrier protein) synthase I and II in that it utilizes CoA thioesters as primers rather than acyl-ACPs | 5.33 | 34189.6 | 1 | 5.12 | 6.14 | 3.85 | 6.77 |
| **Nucleosides and nucleotides biosynthesis and metabolism** | | | | | | | | | | | | |
| 1 | Hypothetical protein, plu3994 | P. luminescens TT01 | | Similar to putative membrane protein YqjD (carboxyl transferase) of E. coli | 7.93 | 11042 | 1 | 4.03 | 0.57 | 2.91 | 1.2 |
| 3 | IS630 family transposase, plu0720 | P. luminescens TT01 | ISPlu3Y | Transposase, IS630 family | 9.33 | 39771.5 | 1 | 0.38 | 0.17 | 0.02 | 0.11 |
| 18 | Nucleoside diphosphate kinase, plu1372 | P. luminescens TT01 | ndk | Catalyzes the formation of nucleoside triphosphate from ATP and nucleoside diphosphate | 5.35 | 15591.8 | 1 | 1.43 | 1.96 | 2.48 | 2.53 |
| 57 | Uracil phosphoribosyl transferase, plu2279 | P. luminescens TT01 | upp | Catalyzes the formation of uracil and 5-phospho-alpha-D-ribofuranose 1-diphosphate from UMP and diphosphate | 5.46 | 22489 | 1 | 1.59 | 2.81 | 1.83 | 0.95 |
| Spot No. | Protein Name/plu | Organism | Gene | Function | Protein PI | Protein MW | Ratio W/W | R2/W | R16/W | R31/W | R33/W |
|---------|-----------------|---------|------|----------|------------|------------|-----------|------|-------|-------|-------|
| 58      | Reverse gyrase   | *Leptospirillum* sp. Group II UBA |         |          | 9.31       | 56458      | 1         | 0.41 | 0.08  | 0.19  | 0.08  |
|         |                  |         |      |          |            |            | Cell wall/membrane biogenesis |       |       |       |       |
| 8       | Karst CG12008-PA, isoform A | *Drosophila melanogaster* | *kst*  | Kst-PA; spectrin beta-heavy chain; beta-H spectrin; | 5.93       | 471351.1   | 1         | 2.20 | 1.8   | 1.33  | 1.16  |
| 43      | Hypothetical protein, plu3994 | *P. luminescens* TT01 | Similar to putative membrane protein YqjD of *E. coli* | 7.93       | 11042      | 1         | 0.21 | 0.05  | 0.1   | 0.08  |
| 5       | Periplasmic chaperone, plu0681 | *P. luminescens* TT01 | hlpA   | Histone-like protein HLP-1 precursor | 9.43       | 18476.8    | 1         | 2.63 | 0.81  | 2.11  | 2.64  |
|         |                  |         |      |          |            |            | Transport and binding proteins |       |       |       |       |
| 10      | Unknown          | *P. luminescens* W14 | From (type III secretion system, partial sequence) GI:27550090 | 6.1        | 16883.6    | 1         | 0    | 1.53  | 0     | 0     |
| 64      | Unknown          | *P. luminescens* W14 | From (type III secretion system, partial sequence) GI:27550090 | 6.1        | 16883.6    | 1         | 0.1  | 0.91  | 0.11  | 0.15  |
| 65      | Unknown          | *P. luminescens* W14 | From (type III secretion system, partial sequence) GI:27550090 | 6.1        | 16883.6    | 1         | 0.11 | 0.93  | 0.11  | 0.16  |
| 11      | Hypothetical protein, plu1886 | *P. luminescens* TT01 | Hypothetical transmembrane protein | 5.88       | 15588.6    | 1         | 1.53 | 2.83  | 1.57  | 0.43  |
| 13      | Macrolide transporter subunit | *P. luminescens* TT01 | macA   | Probable macrolide-specific ABC transporter; confers macrolide resistance via active drug efflux | 40743.2    | 1         | 1     | 0    | 0     | 0     | 0     |
| 17      | Sec-independent protein translocase protein, plu4410 | *P. luminescens* TT01 | tatA   | Sec-independent protein translocase protein | 6.18       | 9289.9     | 1         | 0.76 | 0.81  | 0.72  | 0.23  |
| 42      | Na-binding protein HU-alpha (NS2) (HU-2), plu0492 | *P. luminescens* TT01 | dbhA   | Na-binding protein HU-alpha (NS2) (HU-2) | 9.1        | 9407      | 1         | 2.1  | 2.3   | 0.59  | 0.99  |
|         |                  |         |      |          |            |            | Information and regulatory pathways |       |       |       |       |
| 14      | DNA-binding transcriptional regulator HexR, plu2121 | *P. luminescens* TT01 | *hexR* | Represses the expression of the zwf, eda, glp and gap | 6.97       | 31694.5    | 1         | 2.2  | 1.96  | 1.92  | 0.91  |
| 16      | DnaK transcriptional regulator DksA, plu0876 | *P. luminescens* TT01 | *dksA* | DnaK transcriptional regulator DksA | 5.04       | 17415.8    | 1         | 0    | 0.05  | 0.65  | 0     |
| 20      | Nucleotide-binding protein, plu3881 | *P. luminescens* TT01 | Similar to Unknown protein YajQ of *E. coli* | 5.77       | 18311.4    | 1         | 1.47 | 0.89  | 1.48  | 1.09  |
| 22      | Transcriptional repressor MprA, plu1277 | *P. luminescens* TT01 | mprA   | DNA-binding transcriptional repressor of microcin B17 synthesis and multidrug efflux; negative regulator of the multidrug operon emrAB” | 20365.5    | 1         | 1.67  | 1.18  | 1.38  | 1.09  |
| 27      | Hypothetical protein, plu0318 | *P. luminescens* TT01 | Similar to AidA protein of *Ralstonia solanacearum* | 5.7        | 22068.3    | 1         | 1.03 | 1.68  | 0.86  | 0.87  |
| Spot No. | Protein Name/plu | Organism | Gene | Function | Protein PI | Protein MW | W/W | R2/W | R16/W | R31/W | R33/W |
|---------|-----------------|----------|------|----------|------------|------------|-----|-----|-------|-------|-------|
| 49      | Periplasmic protein disulfide isomerase I, plu0381 | *P. luminescens* TT01 | *dbbA* | Disulfide interchange protein DsbA precursor | 7.7 | 22954.8 | 1   | 3.8 | 1.57  | 3.49  | 3.62  |
| 37      | Protease precursor DegQ, plu4018 | *P. luminescens* TT01 | *degQ* | Protease precursor DegQ | 9.12 | 48028.9 | 1   | 0   | 0     | 0     | 0     |
| 38      | Protease precursor DegQ, plu4018 | *P. luminescens* TT01 | *degQ* | Protease precursor DegQ | 9.12 | 48028.9 | 1   | 0   | 0     | 0     | 0     |

### Energy production and conversion

| Spot No. | Protein Name/plu | Organism | Gene | Function | Protein PI | Protein MW | W/W | R2/W | R16/W | R31/W | R33/W |
|----------|-----------------|----------|------|----------|------------|------------|-----|-----|-------|-------|-------|
| 24       | GD22749 | Dracophila simulans | Dism/ GD22749 | Chromosome segregation ATPases (Cell division and chromosome partitioning): COG1196 | 5.23 | 76326.8 | 1   | 1.39 | 0     | <0.01 | <0.01 |
| 23       | Hypothetical protein, plu2075 | *P. luminescens* TT01 | *deG* | Similar to 3-oxoacyl-[acyl-carrier protein] reductase | 8.55 | 25032.6 | 1   | 0.12 | 0.31  | 0.45  | 0.33  |
| 35       | WblA protein, plu4796 | *P. luminescens* TT01 | *wblA* | Probable lipoplysaccharide biosynthesis protein; Similar to putative UDP-glucose/GDP-mannose dehydrogenase | 5.86 | 48484 | 1   | 2.1  | 0     | 2.07  | 0.05  |
| 40       | Catalase, plu3068 | *P. luminescens* TT01 | *katE* | Catalase | 6.92 | 55509.5 | 1   | 0   | 0     | 0     | 0     |
| 41       | Catalase plu3068 | *P. luminescens* TT01 | *katE* | Catalase | 6.92 | 55509.5 | 1   | 0   | 0     | 0     | 0     |
| 56       | Phosphoglycero mutase, plu1471 | *P. luminescens* TT01 | *gpmA* | Catalyzes the interconversion of 2-phosphoglycerate to 3-phosphoglycerate | 5.62 | 28396.7 | 1   | 1.64 | 2.88  | 2.02  | 3.5   |

### Phage-related proteins

| Spot No. | Protein Name/plu | Organism | Gene | Function | Protein PI | Protein MW | W/W | R2/W | R16/W | R31/W | R33/W |
|----------|-----------------|----------|------|----------|------------|------------|-----|-----|-------|-------|-------|
| 28       | Hypothetical protein, plu3012 | *P. luminescens* TT01 | *katE* | Probable phage protein; Similar to tail fiber assembly protein from bacteriophage | 4.67 | 21467.8 | 1   | 0.68 | 0.88  | 2.36  | 0.44  |
| 29       | Hypothetical protein, plu2035 | *P. luminescens* TT01 | *katE* | Some similarities with putative tail fiber protein of phage | 4.23 | 23641.5 | 1   | 0   | 3     | 0.42  | 0.88  |
| 39       | Hypothetical protein, plu3803 | *P. luminescens* TT01 | *katE* | Some similarities with prophage tail fiber protein | 6.37 | 66850.4 | 1   | 0.23 | 0.5   | 0.1   | 0     |
| 60       | Hypothetical protein, plu3032 | *P. luminescens* TT01 | *katE* | Putative bacteriophage protein; Some similarities with Unknown protein of *Photorhabdus* | 6.08 | 22021.6 | 1   | 1.93 | 2.55  | 2.14  | 4.66  |
| 63       | Hypothetical protein, plu3012 | *P. luminescens* TT01 | *katE* | Probable phage protein; Similar to tail fiber assembly protein from bacteriophage | 4.67 | 21467.8 | 1   | 1.66 | 1.72  | 5.27  | 0.48  |

### Flagellin

| Spot No. | Protein Name/plu | Organism | Gene | Function | Protein PI | Protein MW | W/W | R2/W | R16/W | R31/W | R33/W |
|----------|-----------------|----------|------|----------|------------|------------|-----|-----|-------|-------|-------|
| 31       | Flagellin, plu1954 | *P. luminescens* TT01 | *flic* | Structural flagella protein | 5.19 | 38183.6 | 1   | 0   | 0.1   | 0.87  | 0     |

### Post-translational modification

| Spot No. | Protein Name/plu | Organism | Gene | Function | Protein PI | Protein MW | W/W | R2/W | R16/W | R31/W | R33/W |
|----------|-----------------|----------|------|----------|------------|------------|-----|-----|-------|-------|-------|
| 61       | PTS system, N-acetyl-galactosamine-specific IIB component 2 (EIIb-AGA) (N-acetyl-galactosamine-perme, plu0835) | *P. luminescens* TT01 | *agaV* | Probable PTS system; Highly similar to PTS system, cytoplasmic, N-acetyl/galactosamine-specific | 6.51 | 17774.4 | 1   | 3.87 | 2.18  | 4.31  | 1.5   |
Table 4. Total proteins with altered level of synthesis in the nematicidal-producing and non nematicidal-producing mutants.

| Spot No. | Protein Name/plu | Organism | Gene | Function | Ratio nematicidal-producing strains | Ratio non nematicidal-producing strains |
|----------|-----------------|----------|------|----------|-------------------------------------|----------------------------------------|
|          |                 |          |      |          | W/W | R16/W | R2/W | R31/W | R33/W |
| 26       | 50S ribosomal protein L3, plu4726 | *P. luminescens* TT01 | *rplC* | Binds directly near the 3' end of the 23S rRNA, where it nucleates assembly of the 50S subunit; essential for peptidyltransferase activity; mutations in this gene confer resistance to tiamulin | 1 | 0.41 | 0 | 0 | 0 |
| 4        | ATP-dependent RNA helicase RhlE, plu1511 | *P. luminescens* TT01 | *rhlE* | This helicase is not essential for cell growth | 1 | 0.52 | 0.05 | 0.04 | 0.02 |
| 19       | ATP-dependent RNA helicase RhlE, plu1511 | *P. luminescens* TT01 | *rhlE* | This helicase is not essential for cell growth | 1 | 1.43 | 0 | 0.2 | 0 |
| 5        | Periplasmic chaperone, plu0681 | *P. luminescens* TT01 | *hlpA* | Histone-like protein HLP-1 precursor | 1 | 0.81 | 2.63 | 2.11 | 2.64 |
| 10       | Unknown | *P. luminescens* WI4 | From (type III secretion system, partial sequence) | 1 | 1.53 | 0 | 0 | 0 |
| 64       | Unknown | *P. luminescens* w14 | From (type III secretion system, partial sequence) | 1 | 0.91 | 0.1 | 0.11 | 0.15 |
| 65       | Unknown | *P. luminescens* w14 | From (type III secretion system, partial sequence) | 1 | 0.93 | 0.11 | 0.11 | 0.16 |
| 49       | Periplasmic protein disulfide isomerase I, | *P. luminescens* TT01 | *dsbA* | Disulfide interchange protein DsbA precursor | 1 | 1.57 | 3.8 | 3.49 | 3.62 |
| 2        | Hypothetical protein, plu0661 | *P. luminescens* TT01 | Highly similar to Unknown protein YgdH of *E. coli* | 1 | 0.81 | 2.74 | 2.51 | 1.76 |
| 33       | Hypothetical protein, plu3611 | *P. luminescens* TT01 | Similar to Unknown protein YggE of *E. coli* | 1 | 2.33 | 0 | 0.2 | 0.4 |

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characteristics of phase one bacteria as described: uptake of dye from NBTA and MacConkey agar, production of pH-sensitive pigments, occurrence of inclusion bodies, antibiotic activity, and bioluminescence.

The Effects of the Rif\textsuperscript{R} Mutants on the Growth of H06 Nematodes

13 of 34 Rif\textsuperscript{R} \textit{P. luminescens} LN2 mutants were able to support the growth of H06 IJs, with hermaphrodites containing living juveniles inside and outside after 12 days on the agar plates, while 21 of them were negative for the growth of H06 nematodes. On the bacterial lawns with those mutants or the wild-type, which did not support the nematode production, all the nematodes did not grow beyond adults and died after 7 days.

The Mutation Loci of \textit{rpoB} Gene in the Rif\textsuperscript{R} Mutants

7 positive mutants (LN2-R2, LN2-R6, LN2-R12, LN2-R15, LN2-R28, LN2-R31 and LN2-R33) and 7 negative mutants (LN2-R3, LN2-R5, LN2-R7, LN2-R8, LN2-R11, LN2-R16 and LN2-R25) for H06 nematode growth (Table 2) were randomly selected for \textit{rpoB} gene sequencing. The entire \textit{rpoB} sequences of 14 selected Rif\textsuperscript{R} mutants, wild type strain, LN2-A and LN2-M1 were sequenced at least twice. The \textit{rpoB} gene from all colonies was 4029 bp in length, the same to that of \textit{P. luminescens} subsp. laumontii TT01 [57]. The identity of \textit{rpoB} genes between the wild-type strain of LN2 and TT01 was 96.13\%. All of the 14 Rif\textsuperscript{R} mutants carried mutations in the \textit{rpoB} gene. 10 mutants showed a single nucleotide mutation resulting in an amino acid substitution, and 2 mutants presented two nucleotide mutations resulting in two amino acid substitutions, but only one mutant displayed three nucleotide mutations resulting in two amino acid substitutions (Table 2). No mutation was observed in the \textit{rpoB} gene of Amp\textsuperscript{R} mutant LN2-A and \textit{napA} mutant LN2-M1.

The \textit{rpoB} (P564L) mutation was found in all 7 mutants which produced nematocidal activity against H06 nematodes, but not in the mutants which supported H06 nematode production. While the single mutations of V146F, S112F, Q513K and double mutations of A313D and R529C were detected respectively in the mutants which supported H06 nematode production (Table 2). The single and double mutations resulted in loss of nematocidal activity against H06 nematodes and ability to supported H06 nematode production.

Allelic Exchange Assays

The recombinant LN2-WΔ\textit{rpoB}-LR31 (LN2-W strain containing the mutant \textit{rpoB} allele from LN2-R31) and the LN2-R31Δ\textit{rpoB}-LW (LN2-R31 containing the wild type \textit{rpoB} allele from LN2-W) were selected on Amp\textsuperscript{R} Rif\textsuperscript{E} Te\textsuperscript{c} and Amp\textsuperscript{R} Rif\textsuperscript{E} Te\textsuperscript{c} LB1 agar plates, respectively.

Successful homologous recombination of \textit{rpoB} gene in the recipient strains was verified by randomly selecting three colonies from each recipient and checking their \textit{rpoB} gene sequences by PCR and DNA sequencing. The sequences of \textit{rpoB} gene from three colonies of each recipient were 100\% identical.

The recombinant LN2-WΔ\textit{rpoB}-LR31 showed Rif resistance and lost the nematocidal activity against H06 IJs, while LN2-R31Δ\textit{rpoB}-LW was sensitive to Rif and restored the nematocidal activity. These results clearly indicated that \textit{rpoB} mutation was responsible for the Rif-resistance and the absence of nematocidal activity of LN2-R31.

Insecticidal Activity

The Rif\textsuperscript{R} mutants, including nematocidal-producing LN2-R16 and non nematocidal-producing LN2-R2, LN2-R31 and LN2-R33, together with wild-type of \textit{P. luminescens} LN2 caused 100\% mortality of \textit{G. mellonella} at the concentrations of 1000 CFU/\muL after 24 h, 100 CFU/\muL after 30 h, and 10 CFU/\muL after 36 h. No insect mortality was recorded in the control after 120 h. It appeared that the mutant bacteria also displayed insecticidal activity against \textit{G. mellonella} larvae.

IJ Colonization of the GFP-labelled Mutants

No H06 IJs from the culture with GFP-labeled LN2-R31 mutant contained GFP-labeled bacteria in their intestines. No GFP-labeled bacteria were also observed from the mechanically disrupted H06 IJs. However, the IJs of \textit{H. indica} LN2 from GFP-labeled LN2-R31 mutant contained GFP-labeled bacteria in their intestines. Bacterial colonization of the intestines of IJs is an important process in the nematode-bacterium symbiosis. The present result demonstrated that the mutation of \textit{rpoB} gene restored the nutrient suitability of the LN2 bacteria for the reproduction of H06 nematodes by silencing the nematocidal activity of the bacteria, but did not establish the environment for bacterial colonization of the IJs.

The Proteomic Analysis of the Mutants and Wild-type

The effects of \textit{rpoB} mutations on the nematocidal activity of LN2 bacteria were further investigated by identifying the differentially expressed proteins by 2-DE. The parental and selected \textit{rpoB} mutant strains grown on LB1 agar plates were collected after 48 h. Cells were disrupted and whole cell proteins were separated on 2-DE gels spanning the pH 3–10, silver stained, and analyzed by MS. Protein levels were expressed as percentage volume, which corresponds to the percentage ratio between the volume of a single spot and the total volume of all spots present in a gel. The mean values of spot intensity were calculated using at least three gels. Spots showing more than 15% variation were not considered (Student’s test, with 7 degrees of freedom, p<0.05). Little deviation was observed in the patterns on replica gels.

Approximately 900 spots were revealed on the silver-stained 2-DE patterns of the whole cell proteins from wild type strain LN2-W and the mutant strains of LN2-R16, LN2-R2, LN2-R31 and LN2-R33 (Figure S1, S2, S3, S4, S5). Protein spots were distributed over the 3–10 pH range, with most spots in the 4–7 pH range.

Major differences were detected from different \textit{rpoB} mutants (Figure S1, S2, S3, S4, S5, Table 3). Comparing to the wild type strain, 19, 12 and 13 spots were differentially upregulated, downregulated or missing, respectively, by a factor of at least two in the \textit{rpoB} mutant LN2-R2; 19, 12 and 9 spots in the LN2-R16; 17, 19 and 8 spots in the LN2-R31; and 13, 19 and 14 spots in the LN2-R33.

The spots with intensity changes by a factor of at least two were selected for MALDI-TOF-MS analysis (Table 3), using NCBI website and the PhotoList database (http://genolist.pasteur.fr/PhotoList/).

The proteins identified could be classified into thirteen categories based on functions: (1) ribosomal protein, (2) adaptation conditions, (3) secondary metabolites, (4) metabolism of amino acids and related molecules, (5) nucleosides and nucleotides biosynthesis and metabolism, (6) cell wall/membrane biogenesis, (7) transport and binding proteins, (8) information and regulation pathways, (9) energy production and conversion, (10) phase-related proteins, (11) flagellin, (12) post-translational modification,
and (13) other functions and unknown. A list of the proteins affected by *rpoB* mutation was shown in Table 3.

Proteomic analysis revealed major difference between wild-type strain and RifR mutants, and between nematicidal-producing and non-nematicidal-producing mutants. In all the analyzed *rpoB* mutants, 15 putative proteins (*YbdQ*, *Hep*, *GlyA*, *UreG*, *VioB*, *FabH*, *Ndk*, *Upp*, *Kst*, *MprA*, *DsbA*, *GpmA*, *AgnV*, one bacteriophage protein and one unknown protein) were upregulated, and 11 (*AbiC*, *CipA*, *cipB*, *HcaB*, *ISPP324Y*, *ISPs10*, *YqiB*, *TatA*, *DksA*, *Flc*, and three hypothetical proteins) were downregulated. In particular, the following putative proteins were not detected from all the analyzed *rpoB* mutants: *macA* (probable macrolide-specific ABC transporter, spot 13); *DegQ* (protease precursor, spot 37, 38); and *KatE* (catelase, spot 40, 41).

Interestingly, an unknown function protein *BrecD* (similar to breast cancer type 2 susceptibility protein, spot 21) was not present in the mutants, but present in the wild type strain. It appeared that the absence of these proteins was due to the *rpoB* mutation rather than the antibiotic pressure, because they were also absent from a *namA* disruption mutant of LN2 [41] without *rpoB* mutation in the culture without any rifampin (unpublished data).

Compared with the nematicidal-producing mutant LN2-R16 and wild type strain, four proteins in the non-nematicidal-producing mutants (LN2-R2, LN2-R31 and LN2-R33) were downregulated at least a 2-fold difference in expression, including *RplC* (putative ribosomal protein, spot 26), *RhlE* (putative nucleosides and nucleotides biosynthesis and metabolism protein, spot 4, 19), *NamB* (a putative transport and binding protein from type III secretion system, part of T3SS, spot 10, 64, 65) and a hypothetical protein (similar to unknown protein *YggE* of *E. coli*, spot 33); three proteins including *DsbA* (periplasmic protein disulfide isomerase I involved in involvement and regulatory pathways, spot 49), *HlpA* (periplasmic chaperone involved in cell wall/membrane biogenesis, spot 5), and a hypothetical protein (highly similar to unknown protein *YghH* of *E. coli*, spot 2) were upregulated in the non-nematicidal-producing mutants (LN2-R2, LN2-R31 and LN2-R33) (Table 4, Figure S1, S2, S3, S4, S5). It was suggested that at least these 7 proteins were involved in the nematicidal activity of LN2 bacteria against H06 nematodes.

### Genetic Confirmation of Differentially Expressed Proteins

LN2*AshIE*, LN2*NamB* and LN2*AdhA* mutants showed the typical characteristics of phase one bacteria as the wild type strain. No mutation in *rpoB* gene was observed in these mutants. LN2*AshIE* and LN2*NamB* mutants were able to support the growth of H06 IJs, with hermaphrodites containing living juveniles inside and outside after 12 days on the agar plates, while LN2*AdhA* mutant was negative for the growth of H06 nematodes as the wild type strain. The results confirmed the involvement of these selected genes in the nematicidal activity against H06 nematodes.

### Discussion

In this study, a similar mechanism determining Rif-resistance in *E. coli* and *M. tuberculosis* [3,11,58] was verified in *Photorhabdus* bacteria. Surprisingly, the RifR mutations influenced the nematicidal activity of *P. luminescens* LN2 bacteria against a different nematode, *H. bacteriophora* H06. Furthermore, some but not all *rpoB* mutants of LN2 bacteria lost nematicidal activity against H06 IJs. The *rpoB* mutation was demonstrated to be responsible for the Rif-resistance and the effect on the nematicidal activity in the RifR mutants of LN2. There are fundamental connections between rifampin resistance, RNA polymerase structure and function and global gene expression in the literatures. Rif mutations in *E. coli* affected a wide variety of phenotypes, including altered growth properties and stimulated secondary metabolism [59]. A novel *rpoB* mutation in *B. subtilis* showed a unique spectrum of effects on growth and various developmental events [60]. An *rpoB* mutation in *Streptomyces lividans* activated antibiotic production and reduced growth rate [61]. A spontaneous RifR mutation isolated from *Saccharopolyspora erythraea* stimulated bacterial secondary metabolism and was severely impaired in erythromycin production [62].

To the best of our knowledge, this is the first report that *rpoB* mutations influenced the nematicidal activity of a nematode symbiont on a non-cognate nematode partner. The symbiosis between the entomopathogenic nematodes and their associated bacteria will be also influenced by the *rpoB* mutations. However, how *rpoB* mutations affect this nematicidal activity needs to be further explored.

The mutants exhibited several phenotypes of phase one variant as previously described [47], e.g. absorption of the dye from NBTA and MacConkey agar, production of bioluminescence and occurrence of crystalline inclusion proteins in the cells. It was reported that the nematicidal activity occurred only in phase one of *P. luminescens* LN2 [31]. Apparently, the loss of nematicidal activity in the LN2 mutants against H06 nematodes was not the result of a typical phase variation. The physiological status of symbiotic *Photorhabdus* and *Xenorhabdus* bacteria (such as phase variation, mutants) may influence their fitness for nematode production. As *rpoB* mutations were associated with the nematode growth, screening of *rpoB* mutants of symbiotic bacteria of entomopathogenic nematodes may provide a way to select beneficial *rpoB* mutants by Rif for effective mass production of the nematodes.

One of the important characters in *Photorhabdus* bacteria is their insecticidal activities towards different insects [32–33,35]. The present result indicated that the *rpoB* mutations did not change the expression of the toxin genes, at least in the tested mutants, for the mutants also displayed the insecticidal activity against *G. mellonella* larvae.

Different *rpoB* mutations were associated with their ability to support H06 nematode production. However, the P564L mutation was not associated with the loss of nematicidal activity. The reasons why the mutations affect the physiology and metabolism of the bacterial mutants are not known. The RNA polymerase complex may contact every promoter in the genome, thus any change in critical portions of the enzyme can lead to global changes in gene transcription. Mutations within the Rif binding pocket of *rpoB* gene may alter the structure of RNA polymerase and hence its regulated interaction with specific promoters, and hence physiology and metabolism [63].

Proteomic analysis revealed at least 7 putative proteins including *DsbA*, *RhlE*, *NamB* (a protein from T3SS), *HlpA*, *RplC* and 2 hypothetical proteins *YggE* and *YghH* might be involved in the nematicidal activity. All these proteins may play different roles in different organisms (64–71). In the present study, it was hard to establish the functional relationship among these proteins in the nematicidal activity of LN2 bacteria. Further work is needed to explore this system to understand the molecular mechanism on the trans-specific nematicidal activity of incompatible symbions.
Supporting Information

Figure S1 2-DE map of total cell proteins from *Photorhabdus luminescens* LN2 wild type strain. A representative gel shows the identified differentially expressed protein spots. 350 μg of total cell proteins was loaded onto a 17 cm pH 3–10 NL IPG strip, separated in the second dimension by SDS-polyacrylamide gel electrophoresis on a 12% gel and stained with silver nitrate. (TIF)

Figure S2 2-DE map of total cell proteins from *Photorhabdus luminescens* LN2 Rif<sup>+</sup> mutant LN2-R2. A representative gel shows the identified differentially expressed protein spots. 350 μg of total cell proteins was loaded onto a 17 cm pH 3–10 NL IPG strip, separated in the second dimension by SDS-polyacrylamide gel electrophoresis on a 12% gel and stained with silver nitrate. (TIF)

Figure S3 2-DE map of total cell proteins from *Photorhabdus luminescens* LN2 Rif<sup>+</sup> mutant LN2-R16. A representative gel shows the identified differentially expressed protein spots. 350 μg of total cell proteins was loaded onto a 17 cm pH 3–10 NL IPG strip, separated in the second dimension by SDS-polyacrylamide gel electrophoresis on a 12% gel and stained with silver nitrate. (TIF)

Figure S4 2-DE map of total cell proteins from *Photorhabdus luminescens* LN2 Rif<sup>+</sup> mutant LN2-R31. A representative gel shows the identified differentially expressed protein spots. 350 μg of total cell proteins was loaded onto a 17 cm pH 3–10 NL IPG strip, separated in the second dimension by SDS-polyacrylamide gel electrophoresis on a 12% gel and stained with silver nitrate. (TIF)

Table S1 Oligonucleotide sequences used to generate *Photorhabdus luminescens* LN2 mutant constructs in this study. (DOC)

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Author Contributions

Conceived and designed the experiments: RCH XHQ. Performed the experiments: XHQ XY MXL. Analyzed the data: XHQ RCH. Wrote the paper: RCH XHQ. Obtained the funding, planned and supervised the project: RCH XHQ.

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