Physiological and Molecular Characterization of a Newly Identified Entomopathogenic Bacteria, *Photorhabdus temperata* M1021

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Received: March 29, 2012 / Revised: July 4, 2012 / Accepted: July 26, 2012

The present study concerned the identification and characterization of a novel bacterial strain isolated from entomopathogenic nematodes collected from different regions in Korea. The bacterial isolate M1021 was Gram-negative, bioluminescent, and produced red colonies on MacConkey agar medium. A rod-shaped structure was confirmed by the electron micrograph. Fatty acid composition was analyzed by using the Sherlock MIDI system. The identification was further supported by 16S rDNA sequence analysis, which revealed 96–99% sequence homology with strains of *Photorhabdus temperata*. The location of the isolated strain of *P. temperata* in the phylogenetic tree was confirmed and it was named *P. temperata* M1021. *P. temperata* M1021 exhibited catalase, protease, and lipase activities when grown on appropriate media supplemented with respective substrates. The culture of *P. temperata* M1021 exhibited insecticidal activity against the larvae of *Galleria mellonella* and the activity was the highest after 3–4 days of cultivation with agitating at 28°C under 220 rpm. Antibacterial activity was also observed against *Salmonella Typhimurium* KCTC 1926 and *Micrococcus luteus* KACC 10488.

**Keywords:** Antibacterial activity, entomopathogenic, *Galleria mellonella*, insecticidal toxicity, *Photorhabdus temperata*

*Photorhabdus* spp. are symbiotically associated with entomopathogenic nematodes (EPNs) of the family Heterorhabditidae [7]. The *Photorhabdus–Heterorhabditidae* symbiotic complex passes through three stages during a complete life cycle. In the first symbiotic life cycle stage, the infective form of the EPNs, called infective juveniles (IJJs) [17], take *Photorhabdus* bacteria into their guts and then actively hunt for insect hosts. In the second stage (pathogenic stage), the nematodes enter into the hemocoel of an insect host through its respiratory spiracles or digestive tract [33], and the combined action of the nematodes and bacteria kills susceptible insect hosts within 48 h of infection [26, 33]. In the third stage (replicative stage), the bacterial cells multiply and convert the tissues of the insect cadaver into bacterial biomass using hydrolytic enzymes [8]. The insect dies of septicemia and the IJJs feed on the multiplying symbiotic bacteria, and in this way complete 1–3 generations in the insect host cadaver [19]. Upon depletion of food resources, a large number of IJJs have been produced, which contain colonies of the symbiotic bacteria in their guts, and this symbiotic association is then dispersed in search of new hosts [22]. During infection, the symbiotic bacteria show pathogenicity against the insects [8, 21]. They inhibit the insect immune response by suppressing phospholipase A₂, which catalyzes phospholipids at the sn-2 position to release arachidonic acid [20]. The *Photorhabdus* bacteria also inhibit humoral immunity by down-regulating gene expression of antibacterial peptides including cecropin. They can directly induce apoptosis of insect immunocytes and hemocytes, resulting in complete immune suppression and septicemia [32]. Various antibiotics are synthesized from cultures of symbiotic bacteria whereby xenorhabdins and xenocoumacins are commonly produced by *Xenorhabdus* spp., whereas hydroxystilbenes and anthraquinones are produced by *Photorhabdus* spp. [29]. Currently, three species of *Photorhabdus* have been described to be associated with *Heterorhabditis* nematodes: *P. asymbiotica*, *P. luminescens*, and *P. temperata*. Based on their 16S rDNA sequences and metabolic properties, *P. luminescens* has been divided into
five subspecies [10]. Four out of five subspecies including *P. luminescens* subsp. *luminescens*, *P. luminescens* subsp. *laumondii*, *P. luminescens* subsp. *kayaii*, and *P. luminescens* subsp. *thracensis* have been isolated from *H. bacteriophora*, and one *P. luminescens* subsp. *akhurstii* has been isolated from *H. indica*. Nematode association with *P. temperata* appears to be more diverse. It has been found to be associated with *H. megidis*, *H. zelandica*, *H. marelatus*, and *H. downesi* [12]. *P. temperata* has also been observed to be harbored by *H. bacteriophora* [4]. According to Boemare et al. [7], these reports do not modify the well-established concept of a one-to-one association between EPNs and their symbiotic bacteria, but it does represent a problem for bacterial classification. In fact, DNA–DNA hybridization and 16S rDNA sequence information suggest that *Photorhabdus* is a heterogeneous genus [3], and thus, subspecies definition is necessary [12]. Thus far, two *P. temperata* subspecies have been described: *P. temperata* subsp. *temperata* associated with *H. megidis* [12] and *P. temperata* subsp. *cinerea* associated with *H. downesi* [30]. Indigenous EPNs are perhaps more suitable for inundatory release against local insect pests because they have already adapted to the local climate and other population regulators [24] [5]. In addition, many countries are concerned about the introduction of exotic EPNs because they may have a negative impact on non-target organisms [5].

The main objective of the present study was to identify a novel bacterial strain associated with an endogenous EPN of Korea and investigate its pathogenicity against harmful insects. In addition, we aim to introduce our newly discovered strain into an agriculture field for use as a biocontrol agent.

**Materials and Methods**

**Collection of the Nematodes**

Approximately 200 soil samples were collected from different locations in South Korea. Each sample location consisted of a 50 m² area. Approximately 250 g of soil from each sample location was placed in a pre-sterilized plastic bag and transported to the laboratory. EPNs were isolated from the soil samples through the insect baiting method [7]. The insect bait (*G. mellonella* larvae) was placed in 250 ml plastic containers along with a moist soil sample. The plastic containers containing the bait and soil sample were covered with a lid and maintained at room temperature (20 ± 2°C). Samples were moistened with water if they appeared to be dry at any point during baiting. Larvae bait was checked daily and any dead larvae were removed from the container. After 7–9 days of baiting, the dead larvae (brick red color) were carefully washed with distilled water and put in White traps until emergence of third-stage JIs [16]. Emerging nematodes were collected and then used to infect fresh *G. mellonella* larvae in order to produce nematodes for identification and establishment of bacterial cultures.

**Isolation and Identification of Symbiotic Bacteria**

Symbiotic bacteria were secluded by streaking insect larvae hemolymph on MacConkey agar medium. The culture plates were incubated at 28 ± 2°C for 48 h and then observed for colony growth. Bright-pink colonies grown on MacConkey medium (Becton, Dickinson and Company, USA) were transferred to NBTA plates (nutrient agar with 0.004% triphenyltetrazolium chloride and 0.025% bromothymol blue) and presumed *Photorhabdus* spp. were identified by their characteristic adsorption of blue dye [2]. The bacterial culture in Luria–Bertani (LB) medium was incubated at a temperature range of 28–36°C for 48 h in a shaking incubator at 220 rpm. Bacterial growth was assessed using a spectrophotometer to measure absorbance at OD₆₀₀. The optimal incubation time was determined by incubating the culture broth at 28 ± 2°C for 1 to 8 days in a shaking incubator at 220 rpm. Gram staining was performed by application of the standard Gram stain procedure. The culture broth was deposited on formvar-coated grids for 15 s and followed by staining with 2% uranyl acetate for 25 min. The stained grids were evaluated using a transmission electron microscope.

**Genomic Analysis of the Isolated Bacteria**

Chromosomal DNA was extracted from the symbiotic bacteria using a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA). The extracted DNA segment containing the 16S rDNA gene (approximately 1.5 kb) was amplified by polymerase chain reaction (PCR) using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). The PCR mixture (50 µl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dNTPs, 0.2 pmol of each primer, and *Taq* DNA polymerase (Takara, Japan). Amplification was performed using a protocol consisting of 30 cycles that included 1 min of denaturation at 95°C, 30 s of annealing at 55°C, and 1 min of elongation at 72°C, and a final extension of 5 min at 72°C. The PCR product was then purified (PCR purification kit; Solgent, Daejeon, Korea) and sequencing was conducted by a commercial laboratory (Solgent, Daejeon, Korea). The 16S rDNA gene sequences obtained from the isolated bacterial strain was edited using a multiple sequence editor (DNASIS, Hitachi, America, Ltd) and nucleotide comparison was investigated using the BLASTN program from the National Center for Biotechnology Information (NCBI). The nucleotide sequences were aligned using ClustalW2 from EBI (http://www.ebi.ac.uk/Tools/clustalw2). Pair-wise evolutionary distances were computed using Poisson correction for multiple substitutions. The phylogenetic tree of the isolated strain was constructed using the maximum parsimony method [28] in the Mega 4.0 program.

**Lipase and Protease Activities**

The lipase activity produced by the bacterial isolate was determined using LB agar plates. Two sets of LB media were prepared such that one included Tween 20 and the other included Tween 80. Using autoclaved toothpicks, colonies of bacterial isolate were inoculated on the media. *Escherichia coli* DH5α (negative control) was inoculated on the same media and incubated at 28 ± 2°C for 48 h. LB agar supplemented with 10% (w/v) skim milk was used to determine the protease activity of the bacterial isolate. Using autoclaved toothpicks, bacterial colonies (test sample) were inoculated on culture plates and incubated at 28°C for 48 h. *Bacillus subtilis* 2232 was used as a positive control and *E. coli* DH5α was used as a negative control.
**Antibiotic and Catalase Activities**

An experiment was conducted to investigate the antibiotic activity of the bacterial isolate. Thirty microliter cultures of *S. typhimurium* KCTC 1926 (Korean Collection for Type Cultures) and *M. luteus* KACC 10488 (Korean Agricultural Culture Collection) were spread over the surface of two separate plates containing Tryptone soy agar (TSA) media [tryptone (Bacom) 17.0 g/l, soytryone (Bacom) 3.0 g/l, glucose 2.5 g/l, sodium chloride 5.0 g/l, dipotassium hydrogen phosphate 2.5 g/l, and agar 15 g/l]. The bacterial isolate (test sample) and *E. coli* DH5α (negative control) were inoculated on the *S. typhimurium* and *M. luteus* culture plates. The culture plates were then incubated at 28°C for 48 h. The catalase activity was determined by growing the bacterial strain on tryptone soy agar media. The culture plates were incubated at 28 ± 2°C for 48 h, and then 30 µl of 3% (v/v) H₂O₂ was added to the fully grown colonies of the bacterial isolate. *E. coli* DH5α was used as the positive control and *Streptococcus agalactiae* ATCC13813 (American Type Culture Collection) was used as the catalase negative control.

**Analysis of Fatty Acid Composition**

The fatty acids were analyzed by gas chromatography (Model 6890; Hewlett Packard, Canada) using the Microbial Identification software package [25]. Four different types of reagents were used for fatty acid analysis: Reagent I (45 g sodium hydroxide, 150 ml methanol, and 150 ml distilled water), Reagent II (325 ml certified 6.0 N hydrochloric acid and 275 ml methyl alcohol), Reagent III (150 ml methanol, and 150 ml distilled water), Reagent IV (6.0 N hydrochloric acid and 275 ml methyl alcohol), Reagent V (375 ml distilled water), and Reagent VI (2 ml of Reagent II was added. The tubes were then capped and 2 ml of Reagent II was added. The tubes were then capped and briefly vortexed. After vortexing, the tubes were heated for 10 ± 1 min at 80 ± 1°C. Note that this step was critical in terms of time and temperature. (4) Extraction: Addition of 1.25 ml of Reagent III to the cooled tubes was followed by recapping and gentle tumbling on a clinical rotator for approximately 10 min. The tubes were then unsealed and the aqueous (lower) phase was pipetted out and discarded. (5) Base Wash: Approximately 3 ml of Reagent IV was added to the organic phase remaining in the tubes. The tubes were then unsealed and briefly vortexed. After vortexing, the tubes were heated for 10 ± 1 min at 80 ± 1°C. Note that this step was critical in terms of time and temperature.

**Insect Breeding**

*G. mellonella* larvae were bred from eggs of wax moth (collected from Daegu, Korea) using artificial media. The media ingredients consisted of wheat bran (600 g), rice bran (600 g), yeast extract (4.5 mg), CaCO₃ (2 mg), glycerol (250 ml), water (175 ml), honey (600 ml), and vitamin B-complex (600 mg). All the ingredients, except the honey and vitamins, were mixed thoroughly. Then the mixture and honey were autoclaved in separate containers. After sterilization, the honey, vitamins, and other ingredients were all mixed together. Eggs laid by wax moth on butter paper were added into 150 g of the media and then incubated at 27 ± 1°C and relative humidity 50 ± 5%, whereby the eggs hatched. The small larvae were then transferred to a larger container containing a large amount of medium.

**Toxicity Test of the Bacterial Culture Supernatants**

An experiment was designed to examine the pathogenicity of the isolated bacteria. The bacteria were cultured in LB medium for 48 h at 28 ± 2°C, and then the culture broth was centrifuged at 12,000 rpm in order to harvest the supernatant. Three microcultures of the culture supernatant was injected into the hemocoel of individual *G. mellonella* larvae using a 10 µl Hamilton syringe. Each injected larva was transferred to a 90 mm Petri dish and then incubated at 25 ± 2°C and 50% relative humidity. The larva mortality rate was evaluated after five days. Ten larvae were tested using each bacterial sample and the experiment was repeated three times.

**RESULTS**

**Morphological Characteristics of the Isolate**

The bacterial strain isolated from the EPNs was grown on NBTA at 28 ± 2°C, producing colonies of up to 1.0 mm in diameter after 48 h. The bacterial isolate grew well on all media. The culture plates were incubated at 28 ± 2°C, whereby the eggs hatched. The small larvae were then transferred to a larger container containing a large amount of medium.

**Fig. 1. Biochemical analysis of *P. temperata* M1021.**

(A) Colony morphology of *P. temperata* M1021. (B) Catalase activity of *P. temperata* M1021, forming bubbles in response to the addition of *H₂O₂*. (C) Antibiotic activity of *P. temperata* M1021 against *S. typhimurium*. (D) Antibiotic activity of *P. temperata* M1021 against *M. luteus*. (E) Lipase activity of *P. temperata* M1021 in medium supplemented with Tween. (F) Protease activity of *P. temperata* M1021 in medium supplemented with skim milk. Clear zones around the colonies of *P. temperata* M1021 in Figures C, D, E, and F confirmed the positive results.
the media tested in this study. The colony morphology was granulated, convex, and opaque (Fig. 1A). The bacterial isolate was streaked on NBTA plates and the potential *Photorhabdus* sp. was identified based on phenotypic criteria associated with bioluminescence and colony morphology. The primary colonies appeared as mucoid, convex, and greenish or dark blue with a clear zone due to dye absorption and TTC reduction. Electron micrograph (Fig. 2A) results revealed the rod shape of the bacterial isolate and Fig. 2B illustrates their Gram-negative characteristic.

**Genotypic Characteristics of the Isolate**

The PCR product was purified and sequenced, revealing a length of 1,498 bp. The 16S rDNA sequence from the present study was aligned against a database available at the NCBI. The identification of bacterial isolate was carried out by the maximum parsimony (MP) method. Nine sequences (8 references and 1 clone) were selected for the construction of a phylogenetic tree with 1,000 bootstrap replications. These strains were selected from BLAST search showing maximum sequence homology and query coverage, as well as lowest E values. *Xenorhabdus* was used as the out-group. BLAST search showed that bacterial isolate M1021 has 99.8% sequence homology against the closest relative *Photorhabdus* strain XINach\(^1\), 98% against *P. luminescens* subsp. *luminescens* strain HB\(^1\), 97% against *P. asymbiotica* strain ATCC 43950\(^1\), and 96% against *P. luminescens* subsp. *luminescens* strain ATCC 29999\(^2\). In the dendrogram, bacterial isolate M1021 formed a clade (99% bootstrap support) with *P. temperata* (Fig. 3). On the basis of sequence homology and phylogenetic analysis, isolate M1021 was thus identified as a new strain of *P. temperata*. It was named *Photorhabdus temperata* M1021. The 16S rDNA sequence was deposited into GenBank under the accession number HQ647119.

**Catalase Activity**

The bacterial catalase activity was determined by growing the *P. temperata* M1021 on a TSA plate at 28 ± 2°C for 48 h, and then 30 µl of 3% H\(_2\)O\(_2\) (v/v) was poured over the fully grown colonies (Fig. 1B). Bubble formations over the surface of colonies indicated the presence of catalase. *S. agalactiae* was used as a catalase negative control and *E. coli* DH5\(α\) was used as a catalase positive control.

**Antibiotic Synthesis**

Antibiotic synthesis by *P. temperata* M1021 was confirmed using a simple experiment. Thirty microliter cultures of *S. typhimurium* and *M. luteus* were spread over the surfaces of two different media plates containing tryptone soy agar, and the *P. temperata* M1021 and *E. coli* DH5\(α\) colonies were streaked over the plates. The results (Fig. 1C and 1D) indicated that antibiotics were produced by *P. temperata* M1021, which killed both *S. Typhimurium* and *M. luteus* in their respective culture plates. The secretion of antibiotics functioned to form clear zones around the *P. temperata* M1021 colonies. No clear zones formed around the *E. coli* DH5\(α\) colonies, which were included as a negative control.

**Lipase and Protease Activities**

Lipase and protease activities were found in the *P. temperata* strain M1021. LB media containing either Tween 20 or Tween 80 were used to determine the lipase activity. Results (Fig. 1E) indicated that *P. temperata* M1021 was capable of producing the lipase, which degraded both the Tween 20 and Tween 80 present in the LB media, forming digestion zones around the colonies. The protease activity was determined by incubating the *P. temperata* M1021 on LB agar supplemented with 10% (w/v) skim milk. The formation of clear zones around the *P. temperata* M1021 colonies confirmed the secretion of proteases (Fig. 1F).

**Fatty Acid Composition**

The fatty acid composition of *P. temperata* M1021 was analyzed using a Sherlock MIDI system. The results (Fig. 4) revealed 14 different fatty acids (even and odd numbered, normal, iso, and anteiso; fatty acids are represented by a
binumeric system in which the first number refers to the chain length and the second number refers to the number of double bonds) emerging between 12:0 and 18:0.

**Growth Conditions**

Based on our results, the optimum growth conditions for *P. temperata* M1021 (Fig. 5A) indicated that *P. temperata* M1021 showed best growth activity at 28 ± 2°C and 220 rpm. The effect of the incubation time was also evaluated, with the highest growth rate recorded at the fourth and fifth days of incubation (Fig. 5B). After a long exponential phase, the growth curve for *P. temperata* M1021 reached a stationary phase on the fifth day of incubation. A declination in growth was observed beyond 5 days of incubation.

**Pathogenicity of *P. temperata* M1021**

Approximately 3 µl of the filter extract was injected into fifth instar larvae of *G. mellonella*. The results (Fig. 5C) indicated that *P. temperata* M1021 was highly virulent and caused 100% mortality within 48 h. The LB medium used as a negative control showed no evidence of pathogenicity at any stage of larval incubation. The majority of the larvae injected with the filtered extract died 24–36 h after injection and turned a red-brown color typical of insects infected with *P. temperata*. After a long exponential phase, the growth curve of the *P. temperata* M1021 culture reached a stationary phase on the sixth day of incubation (Fig. 5C). The insecticidal activity contained in the supernatant gradually increased as the incubation time increased, and maximum pathogenicity was noticed in the mid and late stages of the exponential phase (Fig. 5C).

**DISCUSSION**

*Photorhabdus* spp. are Gram-negative, bioluminescent bacteria that live in symbiotic association with soil nematodes (Heterorhabditidae). In nature, the complex formed by the bacterium–nematode has evolved to be an obligate insect pathogen. EPNs require their own symbiotic...
bacteria for successful parasitism against their insect hosts [1]. Two genera of symbiotic bacteria, *Photorhabdus* and *Xenorhabdus*, are found in EPNs [7]. This study was designed to identify and characterize the bacteria isolated from soil nematodes (*Heterorhabditis*) collected from different locations across Korea. Our morphological identification results coincided with positive key characteristics associated with *P. temperata*, including motility, Gram negativity, rod shape, highly bioluminescent, and adsorbing dye on NBTA and MacConkey plates [11, 18, 27]. Optimal bacterial growth for *Photorhabdus* spp. was observed at 28 ± 2°C. This temperature had been previously optimized by Akhurst and Boemare [2]. Morphological characteristics of the bacteria provide some information regarding the identification, but nowadays molecular and phylogenetic approaches have absolutely changed the dynamics of the identification techniques. Genomic DNA sequencing is an objective, reproducible, and rapid technique for identification of bacterial strains. Sequencing of 16S rDNA has gained an immense importance in bacterial identification in recent times, due to the presence of highly conserved sequence of nucleotides in this part of the DNA. Genotypic characteristics strongly supported the hypothesis that the bacterial strain isolated from the EPN belonged to *P. temperata*. Phylogenetic analysis of our discovery confirmed its location in the phylogenetic tree and it was named *P. temperata* M1021. The identification was further confirmed by the fatty acid composition as analyzed by the Sherlock microbial identification system. The fatty acid profile of *P. temperata* M1021 was comparable with the previous analysis of fatty acid composition of *P. temperata* subsp. *temperata* [20, 31]. *P. temperata* releases secondary metabolites such as antibiotics, bacteriocins, lipases, and proteinases, and it is tempting to speculate that the more rigid membranous structures associated with *P. temperata* may facilitate the release of these secondary metabolites [34] as compared with other bacteria. This hypothesis may also explain the increase, instead of the expected decrease, of the structural order of the fatty acids released from *P. temperata* cultured at reduced temperatures. The surface area that a given quantity of phospholipids occupy in their respective membranes decreases at lower temperatures because of the reduced thermal motion in their component acyl chains [23]. This reduction in surface area may induce the opening of additional channels on the cell surface through which different secondary metabolic products may pass. In this manner, the bacteria may maintain proper levels of these compounds in the insect host, thus protecting the cadaver and the bacteria from colonization by other bacterial species as well as providing digestible food for the nematode host at reduced temperatures [13]. The *P. temperata* M1021 discovered during the present study secreted insecticidal toxin into its growth medium. The toxin was extremely lethal when injected into the fifth instar larvae of *G. mellonella*. The hemocoelic injection of the bacterial extract resulted in complete mortality of the insect larvae within 48 h. In typical insect infections, upon entry into the host hemocoel, the nematodes release the symbiotic bacteria in their guts [15, 29]. The presence of the bacterial symbiont is required in order to kill the insect host and to digest the host’s tissues, thereby providing nutrients for nematode growth and development [9]. It was observed during the incubation of *P. temperata* M1021 that after a long exponential phase, the bacteria entered into a stationary phase at the fifth day of the incubation period. The insecticidal activity of *P. temperata* M1021 gradually increased with culture time and reached a peak at the mid and late stages of the exponential phase. The insecticidal activity of entomopathogenic bacteria could be due to the secondary metabolites they produce, such as toxins and enzymes [6, 33]. It is notable that there was a conflict between the results of the present study and previous reports, which observed that culture extracts after 24 h of incubation exhibited the highest insecticidal activity [14]. Considering the combined results, we inferred that the toxins were extracellular in nature and secreted in the mid exponential phase by the strain used in the present study, and secreted in the early exponential phase in the strains used previously [6, 14]. The proposed strain of the present study was named *P. temperata* M1021.

**Acknowledgment**

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0009969) and Korea Ministry of Environment as the Eco-Innovation project.

**References**


