**Photorhabdus heterorhabditis** sp. nov., a symbiont of the entomopathogenic nematode *Heterorhabditis zealandica*

Tiarin Ferreira,1 Carol A. van Reenen,2 Akihito Endo,2 Patrick Tailliez,3,4 Sylvie Pagès,3,4 Cathrin Spröer,5 Antoinette P. Malan1 and Leon M. T. Dicks2

Correspondence
Leon M. T. Dicks
lmtd@sun.ac.za

1Department of Conservation Ecology and Entomology, Stellenbosch University, Private Bag X1, 7602 Matieland, Stellenbosch, South Africa
2Department of Microbiology, Stellenbosch University, Private Bag X1, 7602 Matieland, Stellenbosch, South Africa
3Université Montpellier 2, UMR1333 Diversité, Génotypes & Interactions Microorganismes-Insectes (DGIMI), F-34000 Montpellier, France
4INRA, UMR1333 Diversité, Génotypes & Interactions Microorganismes-Insectes (DGIMI), F-34000 Montpellier, France
5DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstrasse 7B, 38124 Braunschweig, Germany

The bacterial symbionts SF41T and SF783 were isolated from populations of the insect pathogenic nematode *Heterorhabditis zealandica* collected in South Africa. Both strains were closely related to strain Q614 isolated from a population of *Heterorhabditis* sp. collected from soil in Australia in the 1980s. Sequence analysis based on a multigene approach, DNA–DNA hybridization data and phenotypic traits showed that strains SF41T, SF783 and Q614 belong to the same species of the genus *Photorhabdus* with *Photorhabdus temperata* subsp. *cinerea* as the most closely related taxon (DNA–DNA hybridization value of 68%). Moreover, the phylogenetic position of *Photorhabdus temperata* subsp. *cinerea* DSM 19724T initially determined using the *gyrB* sequences, was reconsidered in the light of the data obtained by our multigene approach and DNA–DNA hybridization experiments. Strains SF41T, SF783 and Q614 represent a novel species of the genus *Photorhabdus*, for which the name *Photorhabdus heterorhabditis* sp. nov. is proposed (type strain SF41T = ATCC BAA-2479T = DSM 25263T).

*Photorhabdus* bacteria are symbiotically associated with entomopathogenic nematodes of the genus *Heterorhabditis*, contributing actively to the biological cycle of their host. The Heterorhabditidae family of nematodes consists of obligate insect pathogens. The nematodes and bacteria work together to overcome the immune response of their insect host, thus allowing the bacteria to proliferate. Developing nematodes feed on a mixture of bacteria and bioconverted host tissue, enabling them to produce one to three generations until the food resources in the cadaver are depleted (Koppenhöfer, 2007).

At the time of writing, three species of the genus *Photorhabdus* have been described, *Photorhabdus luminescens* (Thomas & Poinar, 1979, Boemare et al., 1993), *Photorhabdus asymbiotica* and *Photorhabdus temperata* (Fischer-Le Saux et al., 1999). In addition, nine subspecies of *Photorhabdus luminescens*, three subspecies of *Photorhabdus temperata* and two subspecies of *Photorhabdus asymbiotica* have been described. The subspecies of *Photorhabdus luminescens* are: *Photorhabdus luminescens* subsp. akhurstii and *Photorhabdus luminescens* subsp. laemonidii (Fischer-Le Saux et al., 1999), *Photorhabdus luminescens* subsp. caribbeensis and *Photorhabdus luminescens* subsp. hainanensis (Tailliez et al., 2010), *Photorhabdus luminescens* subsp. kayaii (Hazir et al., 2004), *Photorhabdus luminescens* subsp. kleinii (An & Grewal, 2011), *Photorhabdus luminescens* subsp. luminescens (Thomas & Poinar, 1979; Boemare et al., 1993), *Photorhabdus luminescens* subsp. noeniputensis (Ferreira et al., 2013) and ‘*Photorhabdus luminescens* subsp. sonorensis’.

In this study, symbions associated with the entomopathogenic nematode *Heterorhabditis zealandica* Poinar 1990 isolated from South Africa are described as a novel species of the genus *Photorhabdus*. Previously described bacteria associated with *Heterorhabditis zealandica* belong to the species *Photorhabdus temperata* subsp. *tasmaniensis* (Tailliez et al., 2010, Maneesakorn et al., 2011).

Bacterial strain SF41T was obtained from *Heterorhabditis zealandica* from undisturbed soil from Baviaanskloof (33° 41’ 28” S 24° 35’ 23” E), Patensie, in the Northern Cape province of South Africa (Malan et al., 2006). Strain SF783 was obtained from a *Heterorhabditis zealandica* nematode population isolated from a citrus orchard (25° 28’ 4.2” S 31° 3’ 24” E) near Nelspruit, Mpumalanga, South Africa (Malan et al., 2011). Bacteria were obtained from the haemolymph of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) larvae infected with *Heterorhabditis zealandica*. Isolation was achieved by plating on nutrient agar (Biolab) supplemented with 0.004% (w/v) triphehnylterazolium chloride and 0.025% (w/v) bromothymol blue (NBTA) and incubating at 30 °C. Colonies that absorbed the blue colour were submitted for 16S rRNA gene sequencing. *Photorhabdus asymbiotica* subsp. *asymbiotica* DSM 15149T, *Photorhabdus asymbiotica* subsp. *australis* DSM 17609T, *Photorhabdus temperata* subsp. *temperata* DSM 14550T and *Bacillus subtilis* subsp. *subtilis* DSM 10T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. Strain Q614, *Photorhabdus asymbiotica* subsp. *australis* 3265-86T (=DSM 15149T) and *Photorhabdus asymbiotica* subsp. *australis* 9802892T (=DSM 17609T) were from the INRA collection (Montpellier, France).

Strains of species of the genus *Photorhabdus* were routinely cultured in tryptone soy broth (TSB), Luria–Bertani (LB) broth or nutrient broth (NB) (all Biolab). *Escherichia coli* DH5α was cultured in LB (*in vivo* pathogenicity tests), and *E. coli* DH5α transformants (PCR products for DNA sequencing) were grown in LB containing 100 μg ampicillin ml⁻¹. *Bacillus subtilis* subsp. *subtilis* DSM 10T was grown in NB. Strains were stored at −80 °C in TSB containing 40% (v/v) glycerol.

The most important phenotypic characteristics of the genus *Photorhabdus*, as described by Boemare & Akhurst (1988) were investigated. Colony shape and colour were recorded after incubation for 72 h at 30 °C on NBTA.

Growth between 24 °C and 42 °C was determined by inoculating equal cell densities (OD_{660}=0.03) into TSB and NB, respectively. Cell morphology and size were determined using a Leica DM2000 research microscope equipped with Leica Application Suite (LAS), version 3.5.0. Carbohydrate fermentation reactions were recorded using API 20NE and API 50CH E test strips (bioMérieux). The API test strips were inoculated according to the manufacturer’s instructions and reactions were recorded after incubation for 48 h and 10 days at 30 °C. Nitrate reduction and indole production were recorded after 48 h. Carbohydrate assimilation under aerobic conditions were determined using Biolog GN2 microplates (Biolog). The presence or absence of bioluminescence, colony pigmentation, lipase activity on peptone agar containing Tweens 20, 40, 60 and 80, DNase activity, haemolysis of sheep and horse blood, lecinthinase activity, catalase and oxidase production, and ampicillin resistance were determined according to Ferreira et al. (2013a), Somogyi et al. (2002) and Sierra (1957). Antimicrobial activity was tested by using the spot-on lawn method (Akhurst, 1982), with *B. subtilis* subsp. *subtilis* DSM 10T as the target organism.

Bacterial genomic DNA was extracted using a ZR fungal/bacterial DNA kit (Zymo Research). The partial 16S rRNA gene was amplified using primer pair 8F and 1512R (Felske et al., 1997). Partial *recA*, *gyrB*, *dnaN*, *gltX* and *infB* genes were amplified as described previously (Ferreira et al., 2013b). Amplified products were cloned into *E. coli* DH5α and sequenced as described previously (Ferreira et al., 2013a, b). Sequences of the reference strains were obtained from the GenBank database. The sequences of *Xenorhabdus nematophila* ATCC 19061T and *Photorhabdus luminescens* subsp. *laumondii* TT01T were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *Proteus mirabilis* HI4320 was from GenBank (accession no. NC010554). For each bacterial strain, the five individual gene sequences (*recA*, *gyrB*, *dnaN*, *gltX* and *infB*) were concatenated using the SeaView platform (http://doua.prabi.fr/software/seaview). Individual and concatenated sequences were aligned using MUSCLE (Edgar, 2004). Ambiguously aligned blocks were removed using the Gblocks method (Castresana, 2000). The 16S rRNA gene distance phylogenetic tree was calculated using Kimura’s two-parameter model (Kimura, 1980) and the neighbour-joining method (Saitou & Nei, 1987) (16S rRNA gene sequence analysis). The maximum-likelihood phylogenetic trees using protein-coding sequences were carried out with the general time reversible model of substitution and the phyML method (Guindon & Gascuel, 2003). Bootstrap values (Felsenstein, 1988) of more than 50% were indicated at the nodes. MUSCLE, Gblocks, PhyML and bootstrap values were obtained from the phylogeny.fr platform (Dereeper et al., 2008).

DNA–DNA hybridizations were performed independently at DSMZ, Germany, between strain SF41T and *Photorhabdus asymbiotica* subsp. *australis* DSM 17609T, according to Huss et al. (1983), and at LMG, Belgium, between strain
SF41\textsuperscript{T} and \textit{Photorhabdus temperata subsp. cinerea} DSM 19724\textsuperscript{T}, \textit{Photorhabdus asymbiotica} subsp. \textit{asymbiotica} 3265-86\textsuperscript{T}, \textit{Photorhabdus asymbiotica} subsp. \textit{australis} 9802892\textsuperscript{T} and strain Q614. Hybridizations were performed in the presence of 50 % formamide at 39 °C, according to a modification (Goris et al., 1998; Cleenwerck et al., 2002) of the method described by Ezaki et al. (1989).

An in vivo pathogenicity assay was conducted with strains SF41\textsuperscript{T} and SF783. \textit{E. coli} DH5\textalpha{} served as a control (Givaudan & Lanois, 2000). \textit{Galleria mellonella} larvae were reared on an artificial diet (Poinar, 1975) at 28 °C. Bacterial cultures were prepared by inoculating 100 µl culture into 10 ml LB broth and incubating at 30 °C. At an OD\textsubscript{600} of 0.7, 1 ml of each culture was washed in saline [0.85 % (v/v) sodium chloride] and centrifuged three times. Wax moth larvae were surface-sterilized with 70 % (v/v) ethanol using cotton wool, prior to intrahaemocoelic injection. Groups of 20 larvae were injected with 20 µl bacterial cell solution. The number of bacteria in the injected suspension was measured by plating bacteria onto three NBTA plates (Au et al., 2004; Sicard et al., 2006). The experiment was performed on two different dates.

Cells from the blue or blue-green colonies on NBTA (Biolab) were Gram-stain-negative and rod-shaped. Cell sizes were highly variable, ranging from 4.5 × 0.9 µm to 10.4 × 2.0 µm (Fig. S1, available in the online Supplementary Material).

Colonies were 1–3 mm in diameter and blue-green with a red centre on NBTA. Aerobic growth conditions were preferred. Growth temperatures ranged from 24 to 42 °C in NB, and from 24 to 35 °C in TSB. The optimum growth temperature in NB and TSB was 30 °C.

Strains SF41\textsuperscript{T} and SF783 exhibited strong antibacterial activity towards \textit{B. subtilis} subsp. \textit{subtilis} DSM 10\textsuperscript{T}. Strains SF41\textsuperscript{T} and SF783 were bioluminescent, catalase-positive, oxidase-negative and absorbed dye on NBTA and MacConkey agar. Nitrate was not reduced. Lecithinase and DNase activity was observed. Lipase activity was detected on plates containing Tween 20, 40, 60 and 80. Total haemolysis was observed for all strains on sheep and horse blood. Strains SF41\textsuperscript{T} and SF783 were inhibited by ampicillin in solid medium at concentrations higher than 50 µg ml\textsuperscript{-1}, while 25 µg ampicillin ml\textsuperscript{-1} inhibited growth in liquid medium.

A comparison of API results of strains SF41\textsuperscript{T}, SF783, \textit{Photorhabdus asymbiotica} subsp. \textit{asymbiotica} DSM 15149\textsuperscript{T}, \textit{Photorhabdus asymbiotica} subsp. \textit{australis} DSM 17609\textsuperscript{T} and \textit{Photorhabdus temperata} subsp. \textit{temperata} DSM 14550\textsuperscript{T} is shown in Table S1. Strains SF41\textsuperscript{T} and SF783 produced acid from \textit{N}-acetylglucosamine, \textit{D}-fructose, \textit{D}-glucose, glycerol, maltose, \textit{D}-mannose and \textit{D}-xylose; utilized urease; hydrolysed aesculin and gelatin; and assimilated glucose, mannose, \textit{N}-acetylglucosamine, maltose and gluconate. No acid was produced on \textit{D}-arabinose, \textit{D}-sorbitol, trehalose, xyitol or \textit{L}-fucose. Acid production on \textit{D}-ribose, inositol, salicin and gluconate was weak, while malic acid and trisodium citrate were not assimilated. Strain SF783, but not strain SF41\textsuperscript{T}, produced acid from \textit{β}-galactosidase.

Biolog GN2 microplate reactions indicated that strains SF41\textsuperscript{T} and SF783 assimilated \textit{N}-acetyl-\textit{D}-glucosamine, \textit{N}-acetyl-\textit{D}-galactosamine, Tween 40, Tween 80, \textit{D}-fructose, \textit{L}-\textit{d}-glucose, \textit{D}-gluconic acid, \textit{D}-mannose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, \textit{D}-gluconic acid, \textit{p}-hydroxyphenylacetic acid, \textit{L}-alanine glycine, glycyl \textit{L}-aspartic acid, glycyl \textit{L}-glutamic acid, \textit{D}-serine, \textit{L}-serine, inosine, uridine, thymidine, glyceral and \textit{L}-\textit{D}-glycerol phosphate. Strain SF41\textsuperscript{T} also utilized glutamic acid, while strain SF783 was weakly positive for assimilation of citric acid, \textit{D}-alanine and \textit{γ}-amino butyric acid.

Phylogenetic analysis showed that strains SF41\textsuperscript{T}, SF783 (this study) and Q614 (Akhurst & Boemare, 1986) have highly similar 16S rRNA gene (>99.1 %, Fig. S2) and protein-coding (>98.6 %, Fig. 1) sequences, suggesting that these three strains belong to the same species of the genus \textit{Photorhabdus}. The DNA–DNA hybridization value between strains SF41\textsuperscript{T} and Q614 was 78 % (LMG data). This value is higher than the threshold used for delineating bacterial species (>70 %; Wayne et al., 1997) and thus confirms the classification of these three strains in the same species of the genus \textit{Photorhabdus}. Phylogenetic analysis of each protein-coding sequences (Figs S3–S7) showed congruent evolutionary topologies except for \textit{gyrB} (Fig. S3). The trees inferred with the \textit{recA}, \textit{gltX} and \textit{infB} sequences suggest a common ancestor between strains SF41\textsuperscript{T}, SF783, Q614 and \textit{Photorhabdus temperata} subsp. \textit{cinerea} DSM 19724\textsuperscript{T} with \textit{Photorhabdus asymbiotica} as the most closely related species of the genus \textit{Photorhabdus}. Nodes for this clade were well-supported with high bootstrap values (>97 %). This phylogenetic topology was also well-supported by DNA–DNA hybridization data. Indeed, strain SF41\textsuperscript{T} and \textit{Photorhabdus temperata} subsp. \textit{cinerea} DSM 19724\textsuperscript{T} shared 67 % DNA–DNA relatedness (data from LMG), whereas strain SF41\textsuperscript{T} had DNA–DNA hybridization values of only 50.8 % (DSMZ data) with \textit{Photorhabdus asymbiotica subsp. australis} DSM 17609\textsuperscript{T} and 58 % (LMG data) with \textit{Photorhabdus asymbiotica subsp. australis} strain 9802892\textsuperscript{T}, 48 % (LMG data) with \textit{Photorhabdus asymbiotica subsp. australis} strain 3265-86\textsuperscript{T} and 15.6 % (DSMZ data) with \textit{Photorhabdus asymbiotica subsp. australis} DSM 15149\textsuperscript{T}, 40.6 % (DSMZ data) with \textit{Photorhabdus temperata} subsp. \textit{temperata} DSM 14550\textsuperscript{T} (=XLNach\textsuperscript{T}), and 19.0 % (DSMZ data) with \textit{Photorhabdus luminescens} subsp. \textit{luminescens} DSM 3368\textsuperscript{T} (=Hb\textsuperscript{T}). The phylogeny inferred with the \textit{dnaN} sequences (Fig. S5) was also in agreement with those described for the three protein-coding sequences cited above except that the low bootstrap values (<65 %) make uncertain topology within the clade. In contrast to these four protein-coding sequences, the \textit{gyrB} sequences show a significantly different phylogenetic topology. Using the \textit{gyrB} sequences, \textit{Photorhabdus temperata} subsp. \textit{cinerea} strain DSM 19724\textsuperscript{T} was classified in the phylogenetic clade encompassing the six subspecies of \textit{Photorhabdus temperata} described to date (Tóth & Lakatos,
sp. nov. is proposed, with strain SF41T as the type strain of the novel species of the genus Photorhabdus belonging to the same novel species of the genus Heterorhabditis. Strains SF41T, SF783 and Q614 are thus regarded as belonging to different evolutionary histories. Avoid misclassifications linked to genes that were submitted to different evolutionary histories.

These results confirm that a multigene approach must be used to classify novel isolates of the genus Photorhabdus to avoid misclassifications linked to genes that were submitted to different evolutionary histories.

Strains SF41T, SF783 and Q614 are thus regarded as belonging to the same novel species of the genus Photorhabdus, for which the name Photorhabdus heterorhabditis sp. nov. is proposed, with strain SF41T as the type strain. The name pertains to the nematode Heterorhabditis zealandica, from which strains SF41T and SF783 were isolated. Heterorhabditis zealandica populations were also found in association with Photorhabdus temperata subsp. tasmaniensis, which grows at moderate temperatures (Fischer-Le Saux et al., 1999). The species of the nematode host associated with the bacterial symbiont Q614 was not determined.

In the in vivo pathogenicity assay, after 16 h, the mortality of Galleria mellonella larvae injected with strains SF41T and SF783 was higher than 80%, while larvae in the control group (injected with E. coli DH5α) survived. Results from the pathogenicity assay show that these bacterial strains are effective insect pathogens, and have potential to be used in combination with their vector, Heterorhabditis zealandica, as a biological control agent against pest insects.

**Description of Photorhabdus heterorhabditis sp. nov.**

*Photorhabdus heterorhabditis* (he.te.ro.rhab’di.tis. N.L. gen. n. heterorhabditis of the nematode Heterorhabditis).

Cells are Gram-stain-negative, catalase-positive rods. Aerobic growth is preferred, with growth temperatures ranging from 24 °C to 42 °C in NB, and from 24 °C to 35 °C in TSB. Optimal growth in NB and TSB occurs at 30 °C. Colonies on NBTA are blue or blue-green. Acid is produced from N-acetylglucosamine, D-fructose, D-glucose, glyceral, D-mannose, maltose and D-xylene. Able to ferment glucose, hydrolyse arginine, aesculin and gelatin, and produce urease. Assimilates glucose, D-mannose, N-acetylglucosamine, maltose, potassium gluconate (weakly). Nitrate is not reduced.

The type strain is SF41T (=ATCC BAA-2479=DSM 25263T), and shares a DNA–DNA hybridization value of 57% with *Photorhabdus temperata* subsp. cinerea DSM 19724T. An additional strain of the species is SF783 (=DSM 26380=LMG 28118). Both strains were isolated.
from the nematode *Heterorhabditis zealandica* collected in South Africa.

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**References**


