Photorhabdus heterorhabditis subsp. aluminescens subsp. nov., Photorhabdus heterorhabditis subsp. heterorhabditis subsp. nov., Photorhabdus australis subsp. thailandensis subsp. nov., Photorhabdus australis subsp. australis subsp. nov., and Photorhabdus aegyptia sp. nov. isolated from Heterorhabditis entomopathogenic nematodes

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Photorhabdus heterorhabditis subsp. aluminescens subsp. nov., Photorhabdus heterorhabditis subsp. heterorhabditis subsp. nov., Photorhabdus australis subsp. thailandensis subsp. nov., Photorhabdus australis subsp. australis subsp. nov., and Photorhabdus aegyptia sp. nov. isolated from Heterorhabditis entomopathogenic nematodes

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Abstract

Three Gram-stain-negative, rod-shaped, non-spore-forming bacteria, BA1T, Q614T and PB68.1T, isolated from the digestive system of Heterorhabditis entomopathogenic nematodes, were biochemically and molecularly characterized to clarify their taxonomic affiliations. The 16S rRNA gene sequences of these strains suggest that they belong to the Gammaproteobacteria, to the family Morganellaceae, and to the genus Photorhabdus. Deeper analyses using whole-genome-based phylogenetic reconstructions suggest that BA1T is closely related to Photorhabdus akhursti, that Q614T is closely related to Photorhabdus heterorhabditis, and that PB68.1T is closely related to Photorhabdus australis. In silico genomic comparisons confirm these observations: BA1T and P. akhursti 15138T share 68.8% digital DNA–DNA hybridization (dDDH), Q614T and P. heterorhabditis SF41T share 75.4% dDDH, and PB68.1T and P. australis DSM 17609T share 76.6% dDDH. Physiological and biochemical characterizations reveal that these three strains also differ from all validly described Photorhabdus species and from their more closely related taxa, contrary to what was previously suggested. We therefore propose to classify BA1T as a new species within the genus Photorhabdus, Q614T as a new subspecies within P. heterorhabditis, and PB68.1T as a new subspecies within P. australis. Hence, the following names are proposed for these strains: Photorhabdus aegyptia sp. nov. with the type strain BA1T (=DSM 111180T=CCOS 1943T=LMG 31957T), Photorhabdus heterorhabditis subsp. aluminescens subsp. nov. with the type strain Q614T (=DSM 111144T=CCOS 1944T=LMG 31959T) and Photorhabdus australis subsp. thailandensis subsp. nov. with the type strain PB68.1T (=DSM 111145T=CCOS 1942T). These propositions automatically create Photorhabdus heterorhabditis subsp. heterorhabditis subsp. nov. with SF41T as the type strain (currently classified as P. heterorhabditis) and Photorhabdus australis subsp. australis subsp. nov. with DSM17609T as the type strain (currently classified as P. australis).

Species of the bacterial genus Photorhabdus live in a close symbiotic relationship with Heterorhabditis entomopathogenic nematodes (EPNs) [1]. EPNs are soil-inhabiting organisms that parasitize and reproduce inside small arthropods [2, 3]. They colonize their prey by penetrating through the cuticle or natural openings such as the mouth, spiracles...
or the anus, and crawl towards the haemocoel where they release their *Photorhabdus* symbiotic bacterial partners [4]. *Photorhabdus* bacteria multiply, produce immunosuppressors, digestive proteins and secondary metabolites that cause toxemia, septicemia and eventually kill the infected organism [1, 5–8]. The genus *Photorhabdus* was described by Boemare *et al.* in 1993 to include symbiotic bacteria of *Heterorhabditis* EPNs [9]. Since then, several species and subspecies have been described [9–24]. Currently, the genus *Photorhabdus* contains the following 19 species with validly published names: *Photorhabdus akhurstii*, *Photorhabdus asymbiotica*, *Photorhabdus australis*, *Photorhabdus bodei*, *Photorhabdus caribbeanensis*, *Photorhabdus cinerea*, *Photorhabdus hainanensis*, *Photorhabdus heterorhabditis*, *Photorhabdus kayaii*, *Photorhabdus khanii*, *Photorhabdus kleinii*, *Photorhabdus laumondii*, *Photorhabdus luminescens*, *Photorhabdus namnaoenensis*, *Photorhabdus noenieputensis*, *Photorhabdus stackebrandti*, *Photorhabdus tasmaniensis*, *Photorhabdus temperata* and *Photorhabdus thracensis*. *Photorhabdus laumondii* is divided into two subspecies: *P. laumondii* subsp. *laumondii* and *P. laumondii* subsp. *clarkei*; *Photorhabdus khanii* is divided into two subspecies: *P. khanii* subsp. *khanii* and *P. khanii* subsp. *guanajuatensis*; and *Photorhabdus luminescens* is divided into two subspecies: *P. luminescens* subsp. *luminescens* and *P. luminescens* subsp. *mexicana* [16, 17].

Through the years, the taxonomy and nomenclature of this bacterial group have undergone profound changes as more strains have been isolated and new technological capabilities to characterize them have emerged [9, 13, 17, 18, 21]. As a result, the taxonomy of *Photorhabdus* is apparently clearer and whether a bacterial strain represents a new taxon or not is more straightforward to determine than in the past. However, there are still several taxonomic uncertainties, especially regarding bacterial strains that are highly similar to the type strain of the species that they were circumscribed or that were not formally characterized at the taxonomic level. This is the case of at least three bacterial strains: BA1, PB68.1 and Q614 [12, 25–28]. They were isolated from the intestines of *Heterorhabditis* EPNs that were recovered from soil samples in Egypt, Thailand and Australia, respectively. These strains were initially classified as *P. luminescens* BA1, *P. australis* subsp. *australis* PB68.1 and *Xenorhabdus luminescens* Q614. Based on the most recent taxonomy and nomenclature of this bacterial group, these strains are currently classified as *P. luminescens* BA1, *P. australis* subsp. *australis* PB68.1 and *P. heterorhabditis* Q614. This classification relies to a great extent on 16S rRNA and/or housekeeping gene sequences. As it recently became evident that housekeeping gene and 16S rRNA gene sequences provide insufficient information to resolve the phylogenetic relationships of this bacterial group, particularly of closely related taxa, we revisited the phylogenetic relationships of these strains based on whole genome sequences [16, 17]. To this end, we conducted whole genome-based phylogenetic analyses and sequence comparative studies and found out that these three strains actually represent new taxa within the genus *Photorhabdus*. Here we characterize these strains to describe these new taxa and present our taxonomic conclusions.

To physiologically, biochemically and morphologically characterize strains BA1\textsuperscript{T}, PB68.1\textsuperscript{T} and Q614\textsuperscript{T}, we used bacterial cultures from a single primary form colony of each strain. Bacteria primary forms were determined by examining colony characteristics on NBTA plates [Luria–Bertani (LB) agar plates supplemented with 25 mg ml\(^{-1}\) bromothymol blue and 4 mg ml\(^{-1}\) triphenyl-2,3,5-tetrazolium chloride]. The selected primary form colony was further sub-cultured and maintained on LB agar plates at 28°C. Cell morphology was observed under a Zeiss light microscope at a magnification of ×1000, with cells grown for 5 days at 28°C on LB agar plates. Motility was tested on soft agar as described [29]. Cytochrome oxidase production was tested on discs containing N,N-dimethyl-p-phenylenediamine oxidate and α-naphthol according to manufacturer’s conditions (Sigma–Aldrich). Catalase activity was determined by adding a drop of 10% (v/v) H\(_2\)O\(_2\) into 50 µl of a liquid LB-grown, 24-h-old bacterial culture. The ability of bacterial strains to absorb dye was tested by growing the cells on NBTA agar containing bromothymol blue and triphenyl-2,3,5-tetrazolium chloride (Sigma–Aldrich) [30]. Bioluminescence was determined from liquid cultures using a TriStar LB 942 Multimode Microplate Reader (Berthold Technologies). API 20E strips were used according to manufacturer’s instructions (BioMérieux). In this case, strains BA1\textsuperscript{T}, Q614\textsuperscript{T} and PB68.1\textsuperscript{T} were tested in parallel and the results obtained were compared to those published previously and obtained using all *Photorhabdus* type strains [16].

To molecularly characterize strains BA1\textsuperscript{T}, Q614\textsuperscript{T} and PB68.1\textsuperscript{T}, we reconstructed phylogenetic relationships based on 16S rRNA gene sequences and whole genome sequences, and calculated sequence similarity scores. As the full genome sequences of certain type strains are not publicly available, the set of strains used to reconstruct phylogenetic relationships based on 16S rRNA gene sequences, whole genome sequences and biochemical tests are slightly different. Genome sequences of BA1\textsuperscript{T}, Q614\textsuperscript{T} and PB68.1\textsuperscript{T} were obtained as described previously [6, 16, 17, 25]. Whole genome sequence similarities were calculated by the digital DNA–DNA hybridization (dDDH) method using formula 2 of the Genome-to-Genome Distance Calculator (GGDC) web service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) [31–34]. Whole genome-based phylogenetic relationships were reconstructed using the Reference sequence Alignment based Phylogeny builder realphY 1.12 and FastTree 2.1.10 [35–39]. 16S rRNA genes were amplified by PCR and sequenced by Sanger sequencing [40, 41]. The 16S rRNA gene-based phylogenetic relationships were reconstructed using the maximum-likelihood method based on the Kimura two-parameter model in MEGA7 [42, 43]. Sequences were aligned with MUSCLE (version 3.8.31) [44]. The tree with the highest log likelihood (−3554.61) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach.
and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter=0.7354)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 87.62% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 1348 positions in the final dataset. Graphical representation and editing of the phylogenetic tree were performed with the Interactive Tree of Life (version 3.5.1) [45, 46].

Phylogenetic reconstruction based on 16S rRNA gene sequences and 16S rRNA gene sequence similarity calculations indicate that the closest relatives of strain BA1T are *P. akhurstii* DSM 15138T (99.1%) and *P. hainanensis* DSM 22397T (99.1%), the closest relative of strain Q614T is *P. heterorhabditis* SF41T (99.3%) and the closest relative of strain PB68.1T is *P. australis* DSM 17609T (99.7%) (Figs 1 and 2). Lower sequence similarities were found to all validly described species of the genus *Photorhabdus*.

Given the high 16S rRNA gene sequence similarity scores observed and that 16S rRNA and housekeeping gene sequences provide insufficient information to resolve the phylogenetic relationships of this bacterial group, particularly of very closely related species [16, 17], and to fully meet the guidelines of the *ad hoc* Committee on Reconciliation of Approaches to Bacterial Systematics [47] that recommends the dDDH method as the gold standard for bacterial species circumscription, we reconstructed phylogenetic trees based on core genomes and calculated dDDH scores to determine the taxonomic position of these strains. Strain BA1T clusters together with *P. akhurstii* DSM 15138T and *P. hainanensis* DSM 22397T, Q614T clusters together with *P. heterorhabditis* SF41T, and PB68.1T clusters together with *P. australis* DSM 17609T (Fig. 3). The dDDH scores between BA1T and all *Photorhabdus* species were lower than 70% (Fig. 4). The dDDH scores between Q614T and all *Photorhabdus* species, except *P. heterorhabditis* SF41T, were lower than 70%. The dDDH scores between PB68.1T and all *Photorhabdus* species, except *P. australis* DSM 17609T, were lower than 70%. Strains Q614T and *P. heterorhabditis* SF41T share 75.4% dDDH (CI: 72.4–78.1%), and strains PB68.1T and *P. australis* DSM 17609T share 76.6% isDDH (CI: 73.6–79.4%). Given that the thresholds for species and subspecies delimitation are 70 and 79% dDDH, respectively, we propose to classify BA1T as a new *Photorhabdus* species, Q614T as a new subspecies within the species *P. heterorhabditis* and PB68.1T as a new subspecies within the species *P. australis* [16, 32, 47]. Biochemical characterization supports the status of BA1T, Q614T and PB68.1T as new taxa.

![Fig. 1. Maximum-likelihood phylogenetic tree of *Photorhabdus* bacterial strains reconstructed from 1348 nucleotide positions of 16S rRNA gene sequences. Numbers at nodes represent bootstrap values based on 100 replications. Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of gene sequences used for the reconstruction are shown in Table S1 (available in the online version of this article).](image-url)
since they exhibit unique biochemical profiles, which differ from the profiles of other strains from other taxa (Table 1). Citrate utilization, acetoin and indole production, and urease activity are particularly suitable biochemical tests to differentiate the different species of the genus *Photorhabdus* (Table 1).

Based on the results of this polyphasic approach, we propose the creation of *Photorhabdus aegyptia* sp. nov. with the type strain BA1 T (=DSM 111180 T =CCOS 1943 T =LMG 31957 T ), *Photorhabdus heterorhabditis* subsp. *aluimenes* subsp. nov. with the type strain Q614 T (=DSM 111144 T =CCOS 1944 T =LMG 31957 T ) and *Photorhabdus australis* subsp. *thailandensis* subsp. nov. with the type strain PB68.1 T (=DSM 111145 T =CCOS 1942 T ). These propositions automatically create *Photorhabdus heterorhabditis* subsp. *heterorhabditis* subsp. nov. with SF41 T as the type strain (currently classified as *P. heterorhabditis*) and *Photorhabdus australis* subsp. *australis* subsp. nov. with DSM 17609 T as the type strain (currently classified as *P. australis*).

**EMENDED DESCRIPTION OF PHOTORHABDUS AUSTRALIS (AKHURST ET AL. 2004)**

*Machado et al. 2018*

*Photorhabdus australis* (aus. tra'lis. L. fem. adj. *australis*: southern; the type strain of this species was detected in the southern hemisphere).

Maximum temperature for growth is 40 °C. Yellow or no pigment; weakly pigmented. Most isolates are positive for DNase and most are negative for aesculin hydrolysis. Negative for urease and indole production. Most isolates produce acid from gluconate, variable for acid production from aesculin and negative for trehalose. Proteinaceous inclusions are rare. β-Galactosidase weak or positive. Annular haemolysis is variable on sheep blood and horse blood agars. Most isolates are negative for Tween 60 and Tween 80 esterases and most grow on *myo*-inositol. Natural habitat is *Heterorhabditis* EPNs; all isolates were obtained from human clinical specimens in Australia. The type strain of the species is 9802892 T (=CIP 108025 T =ACM 5210 T ).

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**Fig. 2.** Pairwise nucleotide similarities (%) of 16S rRNA gene sequences of *Photorhabdus* strains. 1348 nucleotide positions were analysed. Accession numbers of gene sequences used are shown in Table S1.
**DESCRIPTION OF PHOTORHABDUS AUSTRA LIS SUBSP. AUSTRA LIS SUBSP. NOV.**

*Photorhabdus australis* subsp. *australis* (aus.trai.lis. L. fem. adj. *australis*: southern; the type strain of the species was detected in the Southern Hemisphere).

Maximum temperature for growth is 40°C. Yellow or no pigment; weakly pigmented. Most isolates are positive for DNase and most are negative for aesculin hydrolysis. Negative for urease and indole production. Most isolates produce acid from gluconate; variable for acid production from aesculin and negative for trehalase. Proteinaceous inclusions are rare. Weak β-galactosidase activity. Annular haemolysis is variable on sheep blood and horse blood agars. The type strain of the subspecies is PB68.1^T (=DMS 111145^T=CCOS 1942^T). Whole genome sequences of this strain are available in the NCBI data bank under accession number LOMY01.

**DESCRIPTION OF PHOTORHABDUS AUSTRA LIS SUBSP. THAILAN DENSIS SUBSP. NOV.**

*Photorhabdus australis* subsp. *thailandensis* (thai.land.en’sis N.L. fem. adj. *thailandensis*, from Thailand, the country where the entomopathogenic nematodes that host the type strain were originally collected).

Cells are large rods and motile. Maximum temperature for growth is 40°C. Yellow or orange. Mucoid. Negative for urease, indole, acetoin and H₂S production. Proteinaceous inclusions are rare. Negative for arginine dihydrolase, lysine and ornithine decarboxylase. Positive for β-galactosidase activity. Annular haemolysis is variable on sheep blood and horse blood agars. The type strain is symbiotically associated with *Heterorhabditis indica* EPNs; their natural habitat is the intestines of these nematodes and the insects infected by them. The type strain of the subspecies is PB68.1^T (=DMS 111145^T=CCOS 1942^T). Whole genome sequences of this strain are available in the NCBI data bank under accession number LOMY01.

**EMENDED DESCRIPTION OF PHOTORHABDUS HETERORHABDITIS FERREIRA ET AL. 2014**

*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. Bioluminescence variable. Aerobic growth is preferred, with growth temperatures ranging from 24 to 42°C in nutrient broth (NB) and from 24 to 35°C in tryptic soy broth (TSB). Optimal growth in NB and TSB occurs at 30°C. Colonies on NBTA are blue or blue-green. Indole production negative. Citrate utilization variable. Urease variable. Tryptophan deaminase variable. Acid is produced from N-acetylglucosamine, d-fructose, d-glucose, glycerol, 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 and potassium gluconate (weakly). Nitrate is not reduced. This strain was isolated from *Heterorhabditis*...
zealandica EPNs collected in South Africa. The type strain of the species is SF41\textsuperscript{T} (=ATCC BAA-2479\textsuperscript{T}=DSM 25263\textsuperscript{T}).

**DESCRIPTION OF PHOTORHABDUS HETERORHABDITIS SUBSP. HETERORHABDITIS SUBSP. NOV.**

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

Cells are Gram-stain-negative, catalase-positive rods. Bioluminescent. Aerobic growth is preferred, with growth temperatures ranging from 24 to 42 °C in NB and from 24 to 35 °C in TSB. Optimal growth temperatures in NB and TSB are 24–35 °C for growth and 30 °C for optimal growth. Colonies on NBTA are blue or blue-green. Acid is produced from N-acetylglucosamine, d-fructose, d-glucose, glycerol, d-mannose, maltose and d-xylose. Able to ferment glucose, hydrolyze arginine, aesculin and gelatin, and produce urease. Assimilates glucose, d-mannose, N-acetylglucosamine, maltose and potassium gluconate (weakly). Indole production negative. Citrate utilization negative. Urease negative. Tryptophan deaminase positive. Nitrate is not reduced. This strain was isolated from *Heterorhabditis zealandica* EPNs collected in South Africa. The type strain of the subspecies is SF41\textsuperscript{T} (=ATCC BAA-2479\textsuperscript{T}=DSM 25263\textsuperscript{T}).

**DESCRIPTION OF PHOTORHABDUS HETERORHABDITIS SUBSP. ALUMINESCENS SUBSP. NOV.**

*Photorhabdus heterorhabditis* subsp. *aluminescens* (a.lu.mi.nes’cens. N.L. part. adj. *aluminescens* non-luminescing; for its incapability to produce bioluminescence).

Cells are large motile rods (4.5×1.0–10.0×2.0 µm). Colonies are mucoid, circular, slightly irregular margins, yellow or orange in colour with a diameter of approximately 2 mm after 48 h growth on LB agar. Maximum temperature for growth is 33–34 °C. Negative for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and for H\textsubscript{2}S, indole and acetoin production. Positive for citrate utilization, for urease and gelatinase activity, and for glucose oxidation. Annular haemolysis is observed on sheep blood agar. Natural habitat is *Heterorhabditis* EPNs. The type strain of the subspecies is Q614\textsuperscript{T} (=DSM 111144\textsuperscript{T}=CCOS 1944\textsuperscript{T}=LMG 31959\textsuperscript{T}). Whole genome sequences of this strain are available in the NCBI data bank under accession number JABBCS01.
Table 1. API20E-based phenotypic characters to differentiate all *Photorhabdus* species/subspecies. Strains *P. australis* subsp. *thailandensis* subsp. nov. PB68.1, *P. aegyptia* sp. nov. BA1, and *P. heterorhabditis* subsp. *aluminescens* subsp. nov. Q614 were tested in parallel. The experiment was conducted twice. The obtained results were compared to those published previously [16]. For further information refer to the original studies [9–19,21,23,24,27]. For further information refer to the original studies [9–19,21,23,24,27].

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DESCRIPTION OF PHOTORHABDUS AEGYPTIA SP. NOV.

*Photorhabdus aegyptia* (ae.gyp’t.i.a. L. fem. adj. aegyptia pertaining to Egypt, the country where the entomopathogenic nematodes that host the type strain were originally collected).

Cells are motile, non-spore-forming rods (approx. 1.0 µm wide and 1.5–2.0 µm long), Gram-stain-negative, oxidase-negative and catalase positive. Colonies are mucoid, circular, slightly irregular margins, pale yellow in colour with a diameter of approximately 2 mm after 48 h growth on LB agar and produce light. Good growth occurs on LB at 28–30°C. Negative for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and for H₂S production. Positive for citrate utilization, for urease and gelatinase activity, for glucose oxidation and for indole acetoin production. The type strain is symbiotically associated with *Heterorhabditis indica* EPNs. Their natural habitat is the intestines of these nematodes and the insects infected by them. The type strain is BA1T (≡DMS 111180T=CCOS 1943T=LMG 31957T). Whole genome sequences of this strain are available in the NCBI data bank under accession number JFGV01

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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