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Photorhabdus luminescens subsp. *noenieputensis* subsp. nov., a symbiotic bacterium associated with a novel *Heterorhabditis* species related to *Heterorhabditis indica*

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The bacterial symbiont AM7^T, isolated from a novel entomopathogenic nematode species of the genus *Heterorhabditis*, displays the main phenotypic traits of the genus *Photorhabdus* and is highly pathogenic to *Galleria mellonella*. Phylogenetic analysis based on a multigene approach (16S rRNA, *recA*, *gyrB*, *dnaN*, *gltX* and *infB*) confirmed the classification of isolate AM7^T within the species *Photorhabdus luminescens* and revealed its close relatedness to *Photorhabdus luminescens* subsp. *caribbeanensis*, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis*. The five concatenated protein-encoding sequences (4197 nt) of strain AM7^T revealed 95.8, 95.4 and 94.9 % nucleotide identity to sequences of *P. luminescens* subsp. *caribbeanensis* HG29^T, *P. luminescens* subsp. *akhurstii* FRG04^T and *P. luminescens* subsp. *hainanensis* C8404^T, respectively. These identity values are less than the threshold of 97 % proposed for classification within one of the existing subspecies of *P. luminescens*. Unlike other strains described for *P. luminescens*, strain AM7^T produces acid from adonitol, sorbitol and xylitol, assimilates xylitol and has no lipase activity on medium containing Tween 20 or 60. Strain AM7^T is differentiated from *P. luminescens* subsp. *caribbeanensis* by the assimilation of *N*-acetylglucosamine and the absence of haemolytic activity. Unlike *P. luminescens* subsp. *akhurstii*, strain AM7^T does not assimilate mannitol, and it is distinguished from *P. luminescens* subsp. *hainanensis* by the assimilation of trehalose and citrate, the inability to produce indole from tryptophan and the presence of acetoin production and urease activity. Strain AM7^T (=ATCC BAA-2407^T =DSM 25462^T) belongs to a novel subspecies, and is proposed as the type strain of *Photorhabdus luminescens* subsp. *noenieputensis* sp. nov.

Bacteria of the genus *Photorhabdus* (Boemare *et al.*, 1993) are associated symbiotically with entomopathogenic nematodes of the genus *Heterorhabditis* and are highly pathogenic to insects. The genus *Photorhabdus* belongs to the family *Enterobacteriaceae* and contains three recognized species, *Photorhabdus luminescens*, *P. temperata* and *P. asymbiotica*. *P. luminescens* is further divided into the subspecies *P. luminescens* subsp. *luminescens*, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *laumondii* (Fischer-Le Saux *et al.*, 1999), *P. luminescens* subsp. *kayaii* and *P. luminescens* subsp. *thracensis* (Hazir *et al.*, 2004), *P. luminescens* subsp. *caribbeanensis* and *P. luminescens* subsp. *hainanensis* (Tailliez *et al.*, 2010) and *P. luminescens* subsp. *kleinii* (An & Grewal, 2011). *P. luminescens* subsp. *thracensis* has subsequently been reclassified as *P. temperata* subsp. *thracensis* (Tailliez *et al.*, 2010). *P. temperata* is separated

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *recA*, *dnaN*, *gltX*, *gyrB* and *infB* gene sequences of strain AM7^T are respectively JQ424880–JQ424885.

Six supplementary figures and a supplementary table are available with the online version of this paper.

into *P. temperata* subsp. *temperata* (Fischer-Le Saux *et al.*, 1999), *P. temperata* subsp. *cinerea* (Tóth & Lakatos, 2008), *P. temperata* subsp. *khanii* (synonym *P. temperata* subsp. *stackebrandtii*; An & Grewal, 2010) and *P. temperata* subsp. *tasmaniensis* (Tailliez *et al.*, 2010). *P. temperata* subsp. *stackebrandtii* includes strains GPS11^T (=DSM 23271^T), NC19, Habana and Meg1 (An & Grewal, 2010). Strain NC19^T (=C1^T) was chosen as the type strain of *P. temperata* subsp. *khanii* by Tailliez *et al.* (2010; published online ahead of print in September 2009) and includes strains Habana and Meg1. Thus, *P. temperata* subsp. *stackebrandtii* is a later heterotypic synonym of *P. temperata* subsp. *khanii*. *P. asymbiotica* is divided into *P. asymbiotica* subsp. *australis* and *P. asymbiotica* subsp. *asymbiotica* (Akhurst *et al.*, 2004).

During a survey conducted in citrus orchards in the Mpumalanga province of South Africa, one population of an unknown *Heterorhabditis* species was collected (Malan *et al.*, 2011). We describe here the bacterial symbiont of the genus *Photorhabdus* associated with this unique population, as, at the time of writing, no other population of this unknown species has been collected elsewhere in the world.

Bacterial symbionts from *Heterorhabditis* strain 158-C (18S rRNA gene sequence deposited as GenBank accession no. FJ235075) (Malan *et al.*, 2011) were isolated according to the procedure described by Akhurst (1980). Bacteria were streaked onto NBTA (nutrient agar; Biolab Diagnostics) and the plates were incubated at 25 °C for 48 h. Blue and blue–green colonies were selected randomly from the plates and transferred to tryptic soy broth (TSB; BD). Pure cultures were stored in 40 % (v/v, final concentration) sterile glycerol at −80 °C. Other bacterial strains used in the current study were from the bacterial collection at INRA (Montpellier, France) except for *Bacillus subtilis* DSM 10^T and *P. luminescens* subsp. *kleinii* DSM 23513^T, which were from the DSMZ (Braunschweig, Germany).

Total genomic DNA of an overnight culture of isolate AM7^T was extracted using the ZR fungal/bacterial DNA kit (Zymo Research). DNA of the 16S rRNA gene was amplified as described by Felske *et al.* (1997). The five protein-encoding genes *recA*, *gyrB*, *dnaN*, *gltX* and *infB* were amplified as described by Tailliez *et al.* (2010, 2012). PCRs used were conducted as described previously (Tailliez *et al.*, 2010), using TaKaRa Ex *Taq* (Takara Bio Inc.) together with the supplied 10× Ex *Taq* buffer and dNTP mixture at the concentrations recommended by the manufacturer. Amplified products were purified (QIAquick PCR purification kit; Qiagen) and sequenced (DNA Sequencing Unit, Central Analytical Facility, University of Stellenbosch) using the BigDye Terminator version 3.1 sequencing kit (Applied Biosystems). Sequences were analysed using BLAST (National Center for Biotechnology Information).

Sequences were assembled using the SeqMan module included in the DNASTAR Lasergene software, version 7.0.0. (http://www.dnastar.com). Concatenated gene sequences were obtained using the function 'concatenate' of the SeaView software (http://pbil.univ-lyon1.fr/software/seaview.html). Single-gene and concatenated-gene sequences were aligned using CLUSTAL W (http://www.clustal.org). Neighbour-joining distance (Saitou & Nei, 1987) and maximum-likelihood (Guindon *et al.*, 2010) trees were calculated using the Phylogeny.fr platform (http://www.phylogeny.fr) dedicated to phylogenetic analysis (Dereeper *et al.*, 2008). The neighbour-joining distance algorithm using Kimura's two-parameter model (Kimura, 1980) was used for the 16S rRNA gene sequences. The maximum-likelihood algorithm was used for the five single gene sequences (*recA*, *gyrB*, *dnaN*, *gltX* and *infB*) and the concatenated sequences. Models of evolution were selected using jModelTest to best fit with the data using the AIC (Posada & Crandall, 1998). Sawyer's test for detecting recombination intervals based on the detection of shared patterns of polymorphisms (Sawyer, 1989) was performed with the computer program GENECONV (http://www.math.wustl.edu/~sawyer).

Growth at different temperatures was recorded by measuring the OD_{600}. Ten millilitres of Luria–Bertani (LB) broth (Biolab) was inoculated with 100 µl of an overnight-grown culture and incubated at 26, 30, 37 and 42 °C for 24 h. Catalase activity was determined by adding a drop of 10 % (v/v) H_2O_2 onto a 20-h-old colony on a plate. The ability of strain AM7^T to absorb dye was tested by growing the cells on NBTA (Biolab) containing bromophenol blue and 2,3,5-triphenyltetrazolium chloride (both from Sigma-Aldrich), as described by Koppenhöfer (2007), and on MacConkey agar containing neutral red (Biolab). Bioluminescence was determined by scanning the colonies with the Xenogen *in vivo* imaging system (IVIS; Caliper Life Sciences Inc.). DNase activity was determined by streaking colonies onto DNase test agar (20 g tryptose, 2.0 g DNA, 5.0 g NaCl, 15 g agar and 5 mg methyl green per litre distilled water). Lecithinase activity was determined using nutrient agar plates supplemented with 0.9 % (w/v) NaCl and 10 % (v/v) egg yolk emulsion (Oxoid). Haemolysis was observed by streaking strain AM7^T on sheep-blood agar plates (National Health Laboratory Services, Cape Town, South Africa). Lipase activity was determined by streaking cells onto peptone agar medium supplemented with Tween 20, 40, 60 or 80, as described by Sierra (1957). API 20E and API 20NE characters, acid production from carbohydrates and carbohydrate assimilation using the API50 CH strip were examined as described by the manufacturer (bioMérieux). Reactions were recorded after 24 and 48 h and 10 days of incubation at 30 °C. Resistance to ampicillin was determined in duplicate in microtitre plates by growing serial dilutions of strain AM7^T in LB broth containing 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 µg antibiotic ml^{-1}. Plates were incubated for 48 h at 30 °C. *Escherichia coli* DH5α was used as a negative control. Cell density was determined at 595 nm using a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories). Antibiosis was determined by overlaying colonies of strain AM7^T on nutrient agar medium with 10 ml 0.8 % (w/v) soft agar containing 100 µl of an overnight culture of *B. subtilis* DSM 10^T.

An *in vivo* pathogenicity assay was conducted with strain AM7^T and *E. coli* DH5α as a control as described by Givaudan & Lanois (2000). Larvae of *Galleria mellonella* L. were reared on artificial diet at 28 °C. Ten millilitres of LB broth was inoculated with 100 µl of a growing culture of strain AM7^T or *E. coli* DH5α and incubated at 30 °C to an OD_{595} of 0.7. Bacterial cells were harvested (2×10^2 cells µl^{-1} for AM7^T and 2.1×10^4 cells µl^{-1} for *E. coli* DH5α), washed with 0.8 % (w/v) sterile saline and plated onto NBTA plates and the number of viable cells was determined after 24 h of incubation at 30 °C (Sicard *et al.*, 2006). Twenty *G. mellonella* larvae were surface-sterilized with 70 % (v/v) ethanol and their haemocoel was injected with 20 µl containing bacterial cells of strain AM7^T (4×10^3 c.f.u.) or *E. coli* DH5α (4.2×10^5 c.f.u.), using a BD Micro-Fine syringe.

Phylogenetic position of isolate AM7^T

The 16S rRNA gene sequence of isolate AM7^T was compared with the sequences of representative strains of the different species and subspecies of the genus *Photorhabdus* (Fig. S1, available in IJSEM Online). The sequence of isolate AM7^T clustered with those of the type strains of the various subspecies of *P. luminescens*, indicating that it belongs to the genus *Photorhabdus*, with the closest similarity (98.6 %) to *P. luminescens* subsp. *laumondii* TT01^T.

The accurate phylogenetic position of isolate AM7^T was studied using five protein-encoding sequences (Fig. 1). The *gyrB* and *recA* genes have been used in several phylogenetic studies of *Photorhabdus* and *Xenorhabdus* (Akhurst *et al.*, 2004; An & Grewal, 2010, 2011; Lee & Stock, 2010; Peat *et al.*, 2010; Sergeant *et al.*, 2006; Tailliez *et al.*, 2010, 2012; Tóth & Lakatos, 2008). We also used the *gltX*, *dnaN* and *infB* genes (Tailliez *et al.*, 2010, 2012) as markers with low probability of lateral gene transfer (Lerat *et al.*, 2003). We investigated the *infB* gene instead of the *glnA* gene (Peat *et al.*, 2010), which was not selected by Lerat *et al.* (2003) as a marker with low probability of lateral gene transfer. Nevertheless, *glnA* also appeared to be interesting in resolving many relationships within the phylogeny of *Photorhabdus* (Peat *et al.*, 2010) and should be included in further studies after validation on representatives of all *Photorhabdus* species and subspecies. The *serC* gene was not used in the current study, as was done by Lee & Stock (2010), because the gene in question was shown to be clearly submitted to extensive recombination within *Xenorhabdus*, brother genus to *Photorhabdus*, by Sergeant *et al.* (2006). Whatever the protein-encoding gene used in our study (Figs S2–S6), the phylogenetic analysis confirmed that isolate AM7^T belongs to the species *P. luminescens*. For *dnaN*, *gltX* and *recA*, AM7^T shared a common ancestor with *P. luminescens* subsp. *caribbeanensis* strains HG26 and HG29^T, although low bootstrap values were obtained for *gltX* and *recA* (58 %). For *infB* and *dnaN*, AM7^T was included in a clade consisting of *P. luminescens* subsp. *caribbeanensis*, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis*. For *gyrB*, AM7^T shared a common ancestor with *P. luminescens* subsp. *luminescens* (with a bootstrap value of 70 %), with the position not being in full agreement with that obtained for the other four genes. However, no recombination event was highlighted for the *gyrB* gene using the GeneConv software (Sawyer's test), in contrast to *P. luminescens* subsp. *kayaii* CIP 108428^T, for which recombination events were detected for the *gltX* gene, leading to an atypical phylogenetic position (Tailliez *et al.*, 2010).

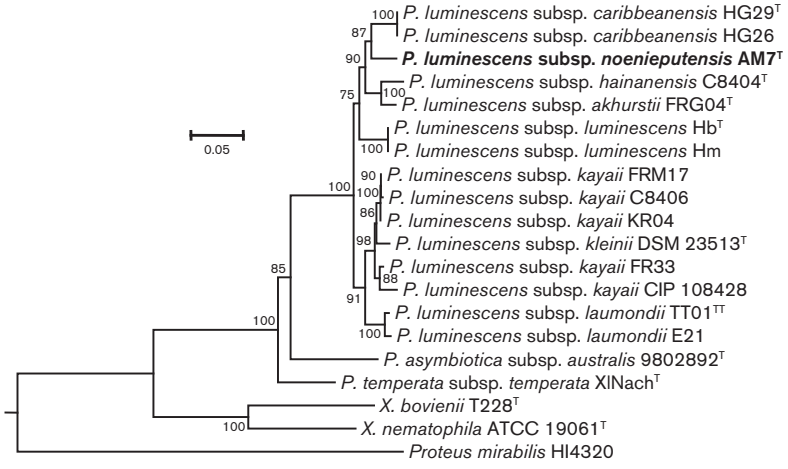

Fig. 1. Maximum-likelihood phylogenetic tree of *P. luminescens* strains reconstructed from concatenated sequences of five protein-encoding genes (*recA*, *gyrB*, *dnaN*, *gltX* and *infB*). Strain AM7^T (in bold) belongs to a monophyletic group including *P. luminescens* subsp. *caribbeanensis*, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis*. The analysis was carried out using the general time reversible model of substitution with gamma-distributed rate heterogeneity and a proportion of invariant sites determined for all five protein-encoding sequences determined by jModelTest to best fit the data using the AIC (Posada & Crandall, 1998). Concatenated sequences from *Xenorhabdus bovienii* T228^T, *Xenorhabdus nematophila* ATCC 19061^T and *Proteus mirabilis* HI4320 were used as an outgroup. Bootstrap values (from 100 replications) (Felsenstein, 1988) more than 50 are shown at nodes. Bar, 5 % divergence.

Table 1. Main phenotypic characters that differentiate *P. luminescens* subspecies

Strains/species: 1, strain AM7^T; 2, *P. luminescens* subsp. *akhurstii* (results for six strains); 3, *P. luminescens* subsp. *caribbeanensis* (*n*=2); 4, *P. luminescens* subsp. *hainanensis* C8404^T; 5, *P. luminescens* subsp. *kayaii* (*n*=6); 6, *P. luminescens* subsp. *laumondii* (*n*=6); 7, *P. luminescens* subsp. *luminescens* (*n*=2). Data for strains other than AM7^T are updated data obtained from Tailliez *et al.* (2010). Results were recorded after 10 days at 30 °C. +, Positive or 90–100 % strains positive; v(+), 50–89 % strains positive; v(−), 11–49 % strains positive; −, negative or 0–10 % strains positive; v, variable; w, weakly positive.

Characteristic	1	2	3	4	5	6	7
Maximum temperature for growth (°C)	37	37–40	38–40	39–40	37–38	35–37	38–40
Pigmentation	+	+	v	−	v(+)	+	+
DNase	−	v(+)	w	−	v(+)	v(+)	+
Arginine dihydrolase	w	−	v	−	v(−)	−	−
Simmons' citrate	+	v	+	+	v(+)	v(+)	+
Urease	+	v(+)	+	−	v(+)	+	v
Indole production	−	v(+)	v	+	v(+)	v	+
Voges–Proskauer reaction	+	−	−	−	v(−)	v(−)	−
Aesculin hydrolysis	+	+	+	+	v	v(+)	v
Acid production from:							
Glycerol	+	+	+	+	v	+	+
Ribose	+	+	+	+	v(+)	v(+)	v
Adonitol	w	−	−	−	−	−	−
Glucose	+	+	+	+	v(+)	+	+
Fructose	+	+	+	+	v	v(+)	+
Mannose	+	+	+	+	v(+)	v(+)	+
Inositol	+	v(+)	w	−	v(−)	v(−)	+
Mannitol	−	v(+)	+	+	−	v(−)	v
Sorbitol	w	−	−	−	−	−	−
N-Acetylglucosamine	+	v	+	+	v	v(+)	+
Aesculin	+	+	+	+	v(+)	v(+)	v
Salicin	w	−	−	−	v(−)	v(−)	−
Maltose	+	+	+	+	v(−)	+	v
Trehalose	+	v	v	+	v(−)	v(+)	w
Xylitol	w	−	−	−	−	−	−
L-Fucose	w	v	+	+	−	−	w
5-Ketogluconate	−	v(−)	v	+	v(−)	v(−)	w
Assimilation of:							
Inositol	+	+	+	+	v(+)	+	+
Mannitol	−	+	+	+	−	−	+
N-Acetylglucosamine	+	+	−	+	+	+	+
Aesculin	+	+	v	+	+	+	v
Trehalose	+	v(+)	+	−	v(+)	v(+)	+
Xylitol	+	v(−)	−	−	−	−	−
L-Fucose	−	v	v	−	−	−	−
Gluconate	+	+	+	+	+	v(+)	+
Caprate	−	v	−	−	v(−)	v(−)	+
L-Malate	−	v(+)	+	−	v(+)	v(+)	+
Citrate	+	v(+)	+	−	+	+	+

The five concatenated sequences of strain AM7^T showed 95.8 % nucleotide sequence identity with those of *P. luminescens* subsp. *caribbeanensis* HG29^T (Fig. 1). Tailliez *et al.* (2010) proposed that, within the three recognized *Photorhabdus* species, *P. asymbiotica*, *P. luminescens* and *P. temperata*, strains that shared less than 97 % nucleotide sequence identity calculated from concatenated sequences of *gyrB*, *recA*, *gltX* and *dnaN* do not belong to the same subspecies. The threshold of 97 % identity is still valid when applied to the concatenation of five sequences (*gyrB*, *recA*, *gltX*, *dnaN* and *infB*; 4197 nt) for differentiating between the *P. luminescens* subspecies recognized previously (Table S1). However, the same rule should be applied with caution after detection of atypical (type) strains and isolates with incongruent single-gene phylogenies, which could lead to misclassifications (e.g. the five concatenated sequences of *P. luminescens* subsp. *kayaii* CIP 108428^T showed only 96.8 % identity, due to its atypical *gyrB* and *gltX* sequences). Analysis of single-gene phylogenies remains essential to avoid erroneous species

assignments. Thus, based on this result, we propose to consider strain AM7^T as a representative of a novel subspecies of *P. luminescens*, *Photorhabdus luminescens* subsp. *noenieputensis* subsp. nov.

Phenotypic characterization of isolate AM7^T

The 15 strains distributed in the different *P. luminescens* subspecies were analysed for their main phenotypic traits (Table 1). Also included in this part of the study were *P. luminescens* subsp. *akhurstii* strains D1 (Australia), EG1 and EG2 (Egypt), IS5 (Israel) and Tetuan (Cuba) and *P. luminescens* subsp. *laumondii* strains HP88 (Utah, USA), HV16 (Australia), NC162 (North Carolina, USA) and K80 (Argentina) in order to increase the robustness of the analysis. These strains were described by Akhurst *et al.* (1996, 2004), Fischer-Le Saux *et al.* (1999), Marokhazi *et al.* (2003) and Peat *et al.* (2010) and were grouped by ribotyping in the laboratory of one of the authors (P. T.). All the *P. luminescens* strains were positive for catalase and negative for lecithinase, oxidase and nitrate reductase activities and were able to assimilate glycerol, ribose, glucose, fructose, D-mannose and maltose. Strain AM7^T can be differentiated from the other *P. luminescens* strains studied by the following specific traits: weak production of acid from adonitol, sorbitol and xylitol, capacity to assimilate xylitol and lack of lipase activity on Tweens 20 and 60. *P. luminescens* subsp. *caribbeanensis* HG29^T and HG26 can be differentiated from strain AM7^T by their annular haemolytic activity on sheep-blood agar and their lack of *N*-acetylglucosamine assimilation. *P. luminescens* subsp. *hainanensis* C8404^T and the majority of the *P. luminescens* subsp. *akhurstii* strains were able to assimilate (6/6 of *P. luminescens* subsp. *akhurstii* strains) and to produce acid (5/6) from mannitol, in contrast to strain AM7^T. Strain AM7^T can also be differentiated from *P. luminescens* subsp. *hainanensis* C8404^T by the assimilation of trehalose and citrate, urease activity, production of acetoin and the lack of indole production from tryptophan.

In vivo pathogenicity assay

After 16 h, all *G. mellonella* larvae that had been injected with strain AM7^T died, whereas larvae of the control group (which had been injected with *E. coli* DH5α) survived. This result confirms the entomopathogenic trait of strain AM7^T.

Description of *Photorhabdus luminescens* subsp. *noenieputensis* subsp. nov.

Photorhabdus luminescens subsp. *noenieputensis* [no.e.ni.e.-put.en′sis. N.L. fem. adj. *noenieputensis* of or belonging to the settlement Noenieput, close to the Namibian border in South Africa, the location of the farm Springbokvlei, the source of the nematode (Malan *et al.*, 2013)].

Cells are Gram-negative, oxidase-negative, catalase-positive and bioluminescent. Colonies are pigmented. Good growth is observed in TSB from 26 °C until the upper limit of 37 °C. Nitrate is not reduced. No DNase or lecithinase activity. A haemolytic reaction is observed when grown on sheep-blood agar plates. Lipolytic activity is observed when grown in the presence of Tweens 40 and 80, but not in the presence of Tween 20 or 60. The bromophenol blue in NBTA and neutral red in MacConkey medium are absorbed by growing cells. Positive for assimilation and acid production from glycerol, ribose, glucose, fructose, mannose, inositol, *N*-acetylglucosamine, aesculin, maltose, trehalose and xylitol. Acid production from adonitol, sorbitol, salicin and L-fucose and assimilation of gluconate and citrate is also observed. Resistant to ampicillin concentrations up to 100 μg ml^{-1}. Growth of *B. subtilis* DSM 10^T is inhibited by cells grown on nutrient agar plates.

The type strain is AM7^T (=ATCC BAA-2407^T =DSM 25462^T), isolated from *Heterorhabditis* strain 158-C.

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