Photorhabdus temperata subsp. *cinerea* subsp. nov., isolated from *Heterorhabditis* nematodes

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During the characterization of symbiotic bacteria of Hungarian entomopathogenic nematode isolates, a number of bacteria (including strain 3107^T) isolated from *Heterorhabditis downesi* and *Heterorhabditis megidis* showed only moderate 16S rRNA gene sequence similarity to the type strains of all described *Photorhabdus* species and subspecies. On the basis of the 16S rRNA gene sequence, the phylogenetic relationships of these isolates were uncertain, because of the low bootstrap values. Using *gyrB* sequences for phylogenetic analysis, these isolates were shown to be part of the species *Photorhabdus temperata*, with clear separation from both Palaearctic and American strains (phylogenetic distances are 6.9 and 7.9 %, respectively). Physiological properties and carbon-source utilization profiles supported the phylogenetic position of these strains; therefore, a novel subspecies, *Photorhabdus temperata* subsp. *cinerea* subsp. nov. is proposed, with the type strain 3107^T (=DSM 19724^T =NCAIM B 02271^T).

The bacterial genus *Photorhabdus*, members of which are associated symbiotically with entomopathogenic nematodes in the genus *Heterorhabditis*, was proposed by Boemare *et al.* (1993) with the transfer of *Xenorhabdus luminescens* (Thomas & Poinar, 1979) to a new genus. After phylogenetic evidence of the taxonomic heterogeneity of *Photorhabdus luminescens* was provided by Szállás *et al.* (1997), Fischer-Le Saux *et al.* (1999) described the three present species of the genus (*Photorhabdus asymbiotica*, *P. luminescens* and *Photorhabdus temperata*), with three subspecies of *P. luminescens* (*P. luminescens* subsp. *akhurstii*, *P. luminescens* subsp. *laumondii* and *P. luminescens* subsp. *luminescens*) and one (illegitimate) subspecies of *P. temperata* (*P. temperata* subsp. *temperata*). Akhurst *et al.* (2004) described two subspecies of *P. asymbiotica* (*P. asymbiotica* subsp. *australis* and *P. asymbiotica* subsp. *asymbiotica*), while Hazir *et al.* (2004) described two further subspecies of *P. luminescens* (*P. luminescens* subsp. *kayaii* and *P. luminescens* subsp. *thracensis*).

During the characterization of symbiotic bacteria of Hungarian entomopathogenic nematode isolates, about 32 % of bacteria (including strain 3107^T) isolated from infective juveniles of *Heterorhabditis* nematodes produced a grey pigment on NA, LB (both from Merck) or TSA (30 g soy peptone, 5 g yeast extract and 12 g agar-agar in 1000 ml water) plates, colouring the culture media. This

trait is not characteristic of any described *Photorhabdus* species or subspecies.

In total, 23 isolates with this special feature were used for morphological and biochemical tests, and three isolates (isolates 3107^T, 3014 and 3240) were selected as typical representatives of the taxon based on colony morphology and carbon-source utilization patterns. The 23 isolates originated from five different geographical locations in Hungary, at least 50 km apart. The nematode hosts were *Heterorhabditis downesi* (eight bacterial isolates) and *Heterorhabditis megidis* (15 bacterial isolates).

Cell morphology was observed under a Zeiss Axiostar light microscope at ×1000 magnification, with cells grown for 3–4 days on TSA at 28 °C. The 16S rRNA and *gyrB* genes of isolates 3107^T, 3014 and 3240 were analysed as described by Tailliez *et al.* (2006) and Akhurst *et al.* (2004), respectively. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007) after multiple alignment of sequences by CLUSTAL_X (Thompson *et al.*, 1997). Distances were calculated using Kimura's two-parameter model, while clustering with the neighbour-joining method was determined by using bootstrap values based on 1000 replications. The details of sequences used for the phylogenetic analyses are listed in Supplementary Table S1 (available in IJSEM Online). Physiological properties and carbon-source utilization profiles were analysed with API 20E strips (bioMérieux) and Biolog GN microplates as described previously (Fischer-Le Saux *et al.*, 1999; Hazir *et al.*, 2004).

Based on analysis of 23 isolates (including 3107^T, 3014 and 3240), all strains grow well on TSA, LB agar and NA plates. Colonies of the strains are highly bioluminescent. Young (1–3 days old) colonies on NA plates are salmon-coloured

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strain 3107^T are EU136626 and EU053168, respectively.

Details of the sequences used for phylogenetic analysis and a 16S rRNA gene sequence-based neighbour-joining tree are available as supplementary material with the online version of this paper.

or light grey. A characteristic grey coloration is visible in the growth medium around older colonies (2–4 days), and the colour of liquid culture media (LB or tryptic soy broth) is also grey. The upper temperature for growth is 34–35 °C (strain 3107^T) or 36–37 °C (strain 3014). The optimal growth temperature is around 28 °C (both on agar plates and in liquid culture), but a phase shift to a secondary form variant (Akhurst, 1980) is very common at this temperature. At lower temperature (20 °C) in liquid culture, the phase shift is rare. In tests with API 20E strips and Biolog GN2 plates, the most important distinguishing biochemical characteristics of the taxon (based on 23 isolates) are the ability to use *myo*-inositol and maltose (distinct from *P. temperata* XINach^T) or xylitol and DL-lactic acid (distinct from all *Photorhabdus* species and subspecies) as a sole carbon source (Table 1). Based on 16S rRNA gene sequences, the separation of this proposed taxon from described species and subspecies of *Photorhabdus* is unambiguous, but the attachment of the genus to the phylogenetic tree is uncertain because of the low bootstrap values (Supplementary Fig. S1). The average similarity to other taxa in the genus is 95.8–97.2 % (Kimura's two-parameter model; 1414 sites). In contrast to the 16S rRNA gene sequence, the *gyrB* gene provides more useful data for investigating intragenomic phylogenies of *Photorhabdus* (Akhurst *et al.*, 2004). Using *gyrB* sequences for phylogenetic analysis, the proposed taxon was shown to be part of the species *Photorhabdus temperata* (Fig. 1). The average similarity to Palaearctic isolates (XINach^T, HL81, HW79 and XLIit; *P. temperata* subsp. *temperata*) is 93.1 %, while the similarity to American isolates (Meg, Habana, C1; *P. temperata*) is 92.1 %. The similarity to the other taxa in the

genus is 88.0–90.7 % (Kimura's two-parameter model; 994 sites).

Creation of this novel subspecies also has the effect of automatically creating the subspecies *Photorhabdus temperata* subsp. *temperata* Fischer-Le Saux *et al.* 1999.

Description of *Photorhabdus temperata* subsp. *cinerea* subsp. nov.

Photorhabdus temperata subsp. *cinerea* (ci.ne.re'a. L. fem. adj. *cinerea* resembling ashes, because this subspecies produces a grey pigment that colours the growing medium and infected insects).

Cells are large rods, 4.8 (2–7) × 1.0 µm. Inclusion bodies are present. Highly bioluminescent. Colonies on nutrient agar are salmon pink or light grey in colour and mucoid; there is grey coloration in the growth medium around the colonies. Adsorbs dye on NBTA and MacConkey plates. Maximum temperature for growth is 34–35 °C (strain 3107^T) or 36–37 °C (strain 3014). Annular haemolysis is present on sheep-blood agar. Negative for *o*-nitrophenyl β-D-galactopyranoside hydrolysis and the Voges–Proskauer test. Gelatinase activity is present. Main biochemical characteristics (including differentiating characters from other *Photorhabdus* species and subspecies) are indicated in Table 1.

The type strain is 3107^T (=DSM 19724^T =NCAIM B 02271^T), isolated from the intestine of the entomopathogenic nematode *Heterorhabditis downesi* collected from a deciduous forest at Ásotthalom, Hungary. Other strains have been isolated from *H. downesi* and *H. megidis*, collected from five different geographical locations in Hungary.

Table 1. Phenotypic characters that differentiate the proposed novel subspecies from described taxa of *Photorhabdus*

Strains: 1, *P. asymbiotica* subsp. *asymbiotica* 3265-86^T; 2, *P. asymbiotica* subsp. *australis* 9802892^T; 3, *P. luminescens* subsp. *akhurstii* FRG04^T; 4, *P. luminescens* subsp. *kayaii* 1121^T; 5, *P. luminescens* subsp. *laumondii* TT01^T; 6, *P. luminescens* subsp. *luminescens* Hb^T; 7, *P. luminescens* subsp. *thracensis* 39-8^T; 8, *P. temperata* XINach^T; 9, 23 strains of *P. temperata* subsp. *cinerea* subsp. nov. [percentages giving a positive reaction (with the exception of the maximum growth temperature); results for isolate 3107^T are in parentheses]. Data are from Akhurst *et al.* (2004) (columns 1 and 2), Hazir *et al.* (2004) (columns 4–7) and this study. +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Maximum growth temperature (°C)	38	40	38	36	36	38	35	34	35
Indole production	–	–	+	+	+	+	–	–	4 (–)
DNase	–	+	–	+	+	–	+	+	100 (+)
Urease	+	–	+	+	+	–	+	–	35 (–)
Tryptophan deaminase	–	–	–	ND	–	–	ND	–	57 (–)
Growth on Simmons' citrate	+	+	+	+	–	+	+	+	87 (+)
Annular haemolysis on sheep-blood agar	+	+	+	+	–	+	+	+	100 (+)
Utilization of:									
L-Fucose	–	–	+	–	–	+	–	+	96 (+)
L-Histidine	+	–	–	–	+	+	+	+	100 (+)
<i>myo</i> -Inositol	–	+	+	+	+	+	–	–	100 (+)
DL-Lactate	–	–	+	–	–	–	–	–	87 (+)
D-Mannitol	–	–	+	–	–	+	–	–	0 (–)
Maltose	+	+	+	+	+	+	+	–	100 (+)
Xylitol	–	–	–	–	–	–	–	–	100 (+)

Fig. 1. Phylogenetic relationships within the genus *Photorhabdus* based on the analysis of *gyrB* gene sequences. Evolutionary history was inferred using the neighbour-joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch lengths of 0.50650502 is shown. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using Kimura's two-parameter method (Kimura, 1980) and are expressed as numbers of base substitutions per site. Positions containing gaps and missing data were eliminated from the dataset, giving a total of 994 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

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