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Short communications

Taxonomic characterisation of *Pseudomonas* strain L48 and formal proposal of *Pseudomonas entomophila* sp. nov.☆

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ABSTRACT

An entomopathogenic, Gram-negative bacterium isolated from a female specimen of the fruit fly *Drosophila melanogaster* was taxonomically characterised. Strain L48^T was strictly aerobic, non-fermentative, oxidase and catalase positive, rod-shaped, and motile due to a polar inserted flagellum. Phylogenetic analysis of the 16S rRNA gene and three other housekeeping genes placed strain L48 ^{T} in the *Pseudomonas putida* phylogenetic group. DNA–DNA hybridisation studies together with phenotypic metabolic tests and MALDI-TOF MS analysis justified the proposal of strain L48^T as a representative of a novel species, for which the name *Pseudomonas entomophila* sp. nov. is proposed. The type strain is deposited in culture collections under accession numbers CCUG 61470^T and CECT 7985^T.

Bacterial strain L48^T was isolated from a female specimen of the fruit fly *Drosophila melanogaster,* collected at Calvaire, (Guadeloupe Island). It was previously described as an entomopathogenic bacterium by Vodovar et al. [27]. Since its first description, numerous studies on strain L48^T have focused on its pathogenicity [24], siderophore production [13] and on genomic and proteomic analysis. Its genome has been completely sequenced, showing a total of 5.888.780 nt [26]. The comparison of the 16S rRNA gene sequence in the databases indicated that strain L48^T belongs to the *Pseudomonas* genus. The three dendrograms deduced from protein coding genes (*rpoD* and *gyrB*) and the 16S rRNA gene sequences indicated that strain L48^T is closely related to *Pseudomonas monteilii* and *Pseudomonas mosselii* species [27]. Because strain L48^T represented an uncharacterised *Pseudomonas* species with some unique entomopathogenic properties, Vodovar and collaborators designated it *Pseudomonas entomophila* [27]. However, *it* has not yet been formally described as a new species and has no standing in the nomenclature. The relevant pathogenic characteristics together with their metabolic versatility warrants a complete taxonomic study in order to consider *P. entomophila* a novel *Pseudomonas* species, with strain L48^T as the type strain. It was deposited in two culture collections under accession numbers CCUG 61470^T and CECT 7985^T.

The type strains of all species in the *Pseudomonas putida* phylogenetic group, as defined in Mulet et al. [14], were included in the present study (Supplementary Table S1). *Pseudomonas alkylphenolia* JCM 16553 [25] belongs phylogenetically to this group. It has no standing in the nomenclature, but was also included. The strains were routinely cultured at 30 °C on LB medium.

For phylogenetic analysis, methods previously described in Mulet et al. [14] were used for DNA extraction, amplification and sequencing by polymerase chain reaction. Primers and accession numbers of sequences used in the analysis are shown in Supplementary Tables S2 and S3 [11,15,19,21,22,29]. A series of individual trees from the 16S rRNA, gyrase beta subunit (*gyrB)*, beta subunit of the RNA polymerase (*rpoB*) and RNA polymerase 70 sigma factor (*rpoD*) partial genes as well as concatenated gene tree of these four genes were constructed as described in Mulet et al. [14,16] and are presented in Fig. 1 and Supplementary Fig. S1. The 16S rRNA, *gyrB*, *rpoB* and *rpoD* gene sequences of *P. entomophila* L48^T were retrieved from the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/) from the genome sequence (NC_008027) [26]. Jukes–Cantor (JC) [7], maximum likelihood (ML) [5] and maximum parsimony (MP) [17]

☆ The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences reported in this study are: HE577792 (strain *P. alkylphenolia* JCM 16553 *gyrB* gene), HE577794 (strain *P. alkylphenolia* JCM 16553 *rpoD* gene), HE577798 (strain *P. alkylphenolia* JCM 16553 *rpoB* gene), HE577795 (strain *P. japonica* JCM 21532^T *rpoD* gene), HE577800 (strain *P. japonica* JCM 21532^T *rpoB* gene), HE577796 (strain *P. taiwanensis* DSM 21245^T *rpoD* gene), HE577797 (strain *P. taiwanensis* DSM 21245^T *rpoB* gene), HE577791 (strain *P. vranovensis* DSM 16006^T *gyrB* gene), HE577793 (strain *P. vranovensis* DSM 16006^T *rpoD*), and HE577799 (strain *P. vranovensis* DSM 16006^T *rpoB* gene).

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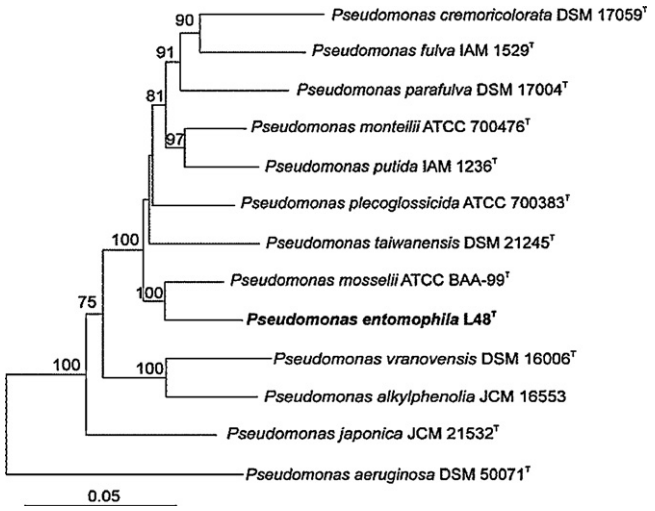

Fig. 1. Phylogenetic tree of *Pseudomonas entomophila* L48^T and phylogenetically close members of *Pseudomonas* based on the analysis of four concatenated genes (16S rRNA, *gyrB*, *rpoD* and *rpoB*; 3723 nt). Distance matrix was calculated by the Jukes–Cantor method. Dendrograms were generated by the neighbour-joining method. *P. aeruginosa* DSM 50071^T was used as outgroup. The bar indicates sequence divergence. Percentage bootstrap values of more than 70% (from 1000 replicates) are indicated at the nodes.

algorithms were used for comparison. *P. entomophila* L48 ^{T} was always located in a separate branch in the *P. putida* group. The lowest 16S rRNA gene similarity within the group was 97.04% for *Pseudomonas vranovensis* DSM 16006^T and *Pseudomonas fulva* IAM 1529^T, and the highest similarity was 99.84% between *Pseudomonas monteilli* ATTC 700476^T, *Pseudomonas taiwanensis* DSM 17004^T and *Pseudomonas plecoglossicida* ATCC 700383^T (Supplementary Table S4). *Pseudomonas mosselli* ATCC BAA-99^T was the closest type strain to *P. entomophila* L48^T (99.76% similarity).

The usefulness of the multilocus sequence analysis (MLSA) of four concatenated genes has been demonstrated previously [14]. A total of 3723 nt from the 4 genes studied (16S rDNA, *gyrB*, *rpoD* and *rpoB*) of the type strains of the species in the *P. putida* group, have been aligned and concatenated. The neighbour-joining tree (Fig. 1) obtained after applying the Jukes–Cantor algorithm indicated that the closest strain to *P. entomophila* L48^T is *P. mosselii* ATCC BAA-99^T (95.55% similarity). The distance with *Pseudomonas aeruginosa* DSM 50071^T, which is the type species of the *Pseudomonas* genus, was 84.73%. The intragroup mean value for members of the *P. putida* group was calculated to be 92.73 ± 3.42%, and 91.19% the minimum similarity between species. Mulet et al. [14] established a similarity of 97% in the MLSA of these four genes as the threshold value for strains in the same species in the genus *Pseudomonas*. The 95.55% similarity obtained between *P. entomophila* L48^T and *P. mosselli* ATCC BAA-99^T indicated that *P. entomophila* L48^T is a member of the *P. putida* group, although it cannot be ascribed phylogenetically to any of the known *Pseudomonas* species.

The *P. entomophila* L48^T chromosome has 5.888.780 nucleotides with a G+C content, deduced from the whole chromosome sequence, of 60 mol% [26]. Compared to the other 20 *Pseudomonas* known genomes, it has an intermediate size; chromosome sizes in *Pseudomonas* species range from 4.56 Mb in *P. stutzeri* A1501 to 7.14 Mb for *Pseudomonas fluorescens* Pf-5 (ftp://ftp.ncbi.nlm.nih.gov/genomes/). The Average Nucleotide Identity (ANIb) [6] within members of the genus was calculated previously, indicating that *P. entomophila* L48^T is related to members of the *P. putida* group, but not to other species in the genus [14].

DNA–DNA hybridisation experiments were performed in duplicate using a method described by Lind and Ursing [12], modified by Ziemke et al. [30]. Reference DNA from strain L48^T, *P. mosselii* and *P. putida* type strains was double-labelled with DIG-1-dUTP and biotin-16-dUTP using a nick translation kit (Boehringer Mannheim). Labelled DNAs were hybridised separately with DNAs from the other type strains (Table 1). The reassociation temperature was 69.3 °C. DNA relatedness is reported as the average percent reassociation relative to the reassociation of the probe DNA to itself. The highest value was obtained in the hybridisation between *P. entomophila* L48^T and *P. putida* IAM 1529^T and was 51%, which is clearly below the accepted species threshold of 70%, confirming that strain *P. entomophila* L48^T was genomically distinct to the type strains of all species within the group.

Morphologically, *P. entomophila* L48^T cells were rod shaped (0.67–1.0 μm wide and 2.2 μm long), motile by means of one polar inserted flagellum (Supplementary Fig. S2) [10] and stained Gram negative following Cowan's method [2]. After incubation for 16 h at 30 °C on LB plates, colonies were circular, beige in colour, flat, with regular edges and approximately 3–4 mm in diameter.

P. entomophila L48^T was able to grow in LB medium at 4 °C, 15 °C, 20 °C, 30 °C and 37 °C in 24 h and showed a slow growth at 42 °C after 36 h incubation. However, no growth was detected at 46 °C or 50 °C. Growth was observed on Nutrient Broth (Merck) in the presence of 0–6% (w/v) NaCl (optimum at 2–3%) and tolerated pH ranging from 3–10 (optimum at 5–9). The oxidase

Table 1
DNA base composition, DNA–DNA hybridisation relatedness values and gene sequence similarities between *Pseudomonas entomophila* L48^T sp. nov. and closely related *Pseudomonas* species.

Bacterial strains	G+C content (mol%)	Reassociation (%)[b] with labelled DNA from			Gene sequence similarities (%) with respect to strain L48^T	
		P. entomophila L48^T	*P. mosselii* ATCC BAA-99^T	*P. putida* IAM 1236^T	16S rRNA	MLSA
P. entomophila L48^T	60	100	48	51	100	100
P. cremoricolorata DSM 17059^T	62[a]	18	32	28	98.7	91.2
P. fulva IAM 1529^T	60[a]	18	35	35	98.3	91.0
P. monteilii ATCC 700476^T	61[a]	23	45	46	99.7	93.2
P. mosselii ATCC BAA-99^T	63[a]	27	100	23	99.8	95.5
P. parafulva DSM 17004^T	60[a]	26	39	38	98.8	91.6
P. plecoglossicida ATCC 700383^T	63[a]	27	39	45	99.7	94.2
P. putida IAM 1236^T	62[a]	26	42	100	98.5	92.5
P. taiwanensis DSM 21245^T	63[a]	36	51[c]	48[c]	99.7	93.0

[a] DNA G+C contents for other closely related *Pseudomonas* type strains were taken from Elomari et al. [4], Nishimori et al. [18], Uchino et al. [23], Dabboussi et al.[3] and Wang et al. [28]. Pooled standard deviations in both experiments were 0.45 and 0.53 (with labelled DNA from *P. entomophila* L48^T), 0.30 and 0.60 (with labelled DNA from *P. putida*^{T}) and 0.30 (with labelled DNA from *P. mosselii*^{T}).
[b] Reassociation values are means of two determinations.
[c] Reassociation values for *P. taiwanensis* DSM 21245^T with labelled DNA from *P. mosselii*^{T} and *P. putida*^{T} were taken from Wang et al. [28].

Table 2
Differential phenotypic characteristics of *Pseudomonas entomophila* L48^T from related *Pseudomonas* type strains.

Characteristic	Strains								
	1	2	3	4	5	6	7	8	9[b]
Cell shape[a]	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility[a]	+	+	+	+	+	+	+	+	+
Flagelation[a]	Single polar	Polar	Polar	ND	Single polar	Polar	Multiple polar	Polar	One or three polar
Fluorescent pigments									
Pseudomonas Agar F	+	+	–	–	+	–	–	+	+
Pseudomonas Agar P	–	–	–	–	–	–	–	–	–
Growth at/on[a]									
5 °C	+	+	+	+	+	+	+	+	+
42 °C	+	–	–	–	–	–	–	–	+
pH 4	–	–	–	–	+	–	–	–	+
6% NaCl	+	+	+	+	+	+	–	–	+
7% NaCl	–	+	+	–	–	+	–	–	–
8% NaCl	–	–	+	–	–	–	–	–	–
Activity of enzymes (API 20NE test)									
Reduction of nitrate	–	–	–	–	–	–	+	–	–
Presence of arginine dihydrolase	+	–	–	+	+	+	+	+	+
Urease	–	–	–	–	+	–	–	–	–
Hydrolysis of gelatin	+	–	–	–	+	–	–	–	–
Growth on (API 20NE test)									
Arabinose	–	–	–	+	–	–	–	–	+
Mannose	+	–	–	–	+	+	–	–	–
Manitol	+	–	–	–	+	–	–	–	–
N-acetyl-glucosamine	+	–	–	–	+	–	–	–	–
Phenylacetic acid	+	–	–	+	+	+	+	+	+
Carbon sources (Biolog GN2)									
α-Cyclodextrin	–	–	–	w	–	–	–	–	
Dextrin	–	–	–	+	–	–	+	w	–
Glycogen	w	+	w	+	–	+	+	+	–
Tween 40	+	+	+	+	+	+	–	+	–
N-acetyl-D-glucosamine	+	–	–	–	+	–	–	–	–
Adonitol	–	–	–	–	–	+	–	–	–
L-Arabinose	+	+	+	+	+	–	+	+	+
D-Arabitol	+	–	–	–	+	–	–	–	–
D-Cellobiose	–	–	w	–	–	–	+	–	–
i-Erythritol	–	–	–	+	–	–	+	–	–
D-Fructose	+	+	–	+	+	–	w	+	+
L-Fucose	w	–	–	–	–	–	–	–	–
α-D-Glucose	+	+	+	–	+	+	+	+	+
D-Mannitol	+	–	–	–	+	–	–	–	–
D-Mannose	+	–	+	w	+	+	+	+	–
D-Melibiose	–	–	w	+	–	–	+	+	–
β-Methyl-D-glucoside	–	–	w	+	–	–	+	w	–
D-Psicose	–	–	–	+	+	–	–	+	–
D-Raffinose	–	–	–	–	–	–	w	–	–
L-Rhamnose	–	–	–	w	–	–	+	–	–
D-Sorbitol	–	–	–	–	–	–	+	–	–
D-Trehalose	–	–	–	–	–	–	+	–	–
Turanose	–	–	–	+	–	–	+	–	–
Xylitol	–	–	–	–	–	–	w	–	–
Mono-methyl-succinate	+	+	+	+	w	+	+	+	+
Formic acid	+	+	–	+	+	+	+	+	–
D-Galacturonic acid	–	–	–	+	–	–	–	+	+
D-Glucosaminic acid	–	–	–	+	–	–	w	+	–
D-Glucuronic acid	–	–	–	+	–	–	w	+	+
α-Hydroxy butyric acid	+	+	+	w	+	+	+	+	+
γ-Hydroxy butyric acid	+	–	–	–	–	–	–	–	+
p-Hydroxy phenylacetic acid	+	–	+	w	+	+	w	+	–
Itaconic acid	–	–	+	w	–	–	+	+	–
α-Keto butyric acid	+	–	+	–	w	+	+	+	+
α-Keto valeric acid	+	–	–	w	+	+	+	+	+

Species: 1, *P. entomophila* L48^T; 2, *P. cremoricolorata* DSM 17059^T; 3, *P. fulva* IAM 1529^T; 4, *P. monteilii* ATCC 700476^T; 5, *P. mosselii* ATCC BAA-99^T; 6, *P. parafulva* DSM 17004^T; 7, *P. plecoglossicida* ATCC 700383^T; 8, *P. putida* IAM 1236^T and 9, *P. taiwanensis* DSM 21245^T. In the API 20NE test, all the type strains were positive for growth on glucose, potassium gluconate, capric acid, malic acid and trisodium citrate, were negative for indole production, glucose fermentation, hydrolysis esculin and (-galactosidase and growth on maltose and adipate. In the oxidase and catalase tests all the type strains were positive. In the Biolog GN2 test, all strains were positive for the following characteristics: Tween 80, methyl pyruvate, acetic acid, cis-aconitic acid, citric acid, D-gluconic acid, β-hydroxy butyric acid, α-keto glutaric acid, D,L-lactic acid, propionic acid, succinic acid, bromo succinic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-leucine, L-proline, L-serine, L-threonine, D,L-carnitine, γ-amino butytic acid, putrescine, but were negative for: N-acetyl-D-galactosamine, D-galactose, gentiobiose, m-inositol, α-D-lactose, lactulose, maltose, sucrose, D-galactonic acid lactone, sebacic acid, thymidine, glucose-1-phosphate. Characteristics are scored as: +, positive reaction; –, negative reaction; w, weakly positive reaction and ND, not determined.

[a] Cell shape, motility, flagellation and growth at/on 5 °C, 42 °C, pH 4, 6% NaCl, 7% NaCl and 8% NaCl for other closely related *Pseudomonas* type strains were taken from Elomari et al. [4], Nishimori et al. [18], Uchino et al. [23], Dabboussi et al. [3] and Wang et al. [28].

[b] The results of the API 20NE and Biolog GN2 tests for *P. taiwanensis* DSM 21245^T were taken from Wang et al. [28].

test was performed with the cytochrome oxidase test paper. The catalase activity was detected by adding 3% hydrogen peroxide solution to resting cells and examining the production of bubbles. Production of a fluorescent pigment was tested on King B medium (*Pseudomonas* agar F, Difco), and pyocyanin production was detected with King A medium (*Pseudomonas* agar P, Difco) [9]. Differential phenotypical characteristics are indicated in Table 2.

Additional physiological and biochemical characteristics were tested using API 20NE, (bioMérieux) and Biolog GN2 microplates (MicroLog System) according to the manufacturer's instructions. The results are shown in Table 2. API 20NE test results indicated that *P. entomophila* L48^T differed from *P. mosselii* ATCC BAA-99^T only in urease activity, which is negative for *P. mosselii* and positive for all other *Pseudomonas* of the group.

All type strains in the *P. putida* group were negative for gelatinase, manitol and N-acetyl-glucosamine, with the exception of strain L48^T and *P. mosselii* ATCC BAA-99^T. The API 20NE and Biolog tests indicated that *P. entomophila* L48^T was positive in the ability to use gamma-hydroxy butyric acid as a carbon source, whereas *P. mosselli* ATCC BAA-99^T was negative. *P. entomophila* L48^T was urease negative and did not use D-psicose as a carbon source, and *P. mosselli* was positive in both tests. As shown in Table 2, *P. entomophila* L48^T differed in three biochemical tests from *P. mosselli* and in more than nine with the other strains of the *P. putida* group. *P. entomophila* L48^T also differed in 10 different phenotypic characteristics with *Pseudomonas parafulva* DSM 17004^T; 14 with *P. fulva* IAM 1529^T, *P. putida* IAM 1236^T, *Pseudomonas cremoricolorata* DSM 17059^T and *P. taiwanensis* DSM 21245^T; 19 with *P. plecoglossicida* ATCC 700383^T; and 20 with *P. monteilli* ATCC 700476^T.

To determine chemotaxonomical markers, a Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) analysis of *P. entomophila* L48^T and its closely related type strains was performed at Anagnosteg GmbH (Germany) [8] as described by Scotta et al. [20]. The profile of peaks obtained for each species was analysed and compared using the software BGP database available at the website http://sourceforge.net/projects/bgp. A dendrogram was generated using the Permutmatrix programme, applying average linkage method (UPGMA hierarchical clustering) and Pearson's distance correlation [1]. The duplicate or quadruplicate whole cell spectra profiles for each strain analysed clustered at least with a 60% similarity. Spectra of two different type strains were less than 50% similar. In the MALDI-TOF MS analysis *P. entomophila* L48^T showed twenty-two single m/z peaks (supermass values) not present in the other strains. These data further support the conclusion that *P. entomophila* strain L48^T is a distinct species and is separate from all other *Pseudomonas* species, even at the level of expression of the most abundant cellular proteins as shown in Supplementary Fig. S3.

Additionally, gas chromatography of cell fatty acids methyl esters composition (CFA) was determined at the CCUG (Culture collection, University of Göteborg, Sweden) using a standardized protocol, similar to that of the MIDI Scherlock MIS system (http://www.ccug.se/pages/CFAmethod2008.pdf). Major CFAs for strain L48^T were C16:0 (25.1%), C16:1 w7c (14.7%), C17:0 CYCLO (13.1%); the summed feature 2 (C18:1 w7c/12t/9t; 12.2%), C10:0 3-OH (9.4%), C12:0 2-OH (6.8%), C12:0 3-OH (5%); and the unidentified CFA with ECL 10.651 (4.4%); all other detected CFAs values were less than 2.0%. Comparative results for close related *Pseudomonas* type strains are given in Supplementary Table S5.

Considering the phylogenetic, chemotaxonomic, genomic and phenotypic characteristics, we propose *P. entomophila* strain L48^T as the type strain of the new species *Pseudomonas entomophila* sp. nov.

Formal description of *Pseudomonas entomophila* sp. nov.

Pseudomonas entomophila (en.to.mo'.phi.la. Gr. n. *entomon*, insect; N.L. fem. adj. *phila*, from Gr. fem. adj. *philê*, loving; N.L. fem. adj. *entomophila*, insect loving, isolated from *D. melanogaster*).

Cells are Gram-negative rods that are 0.67–1.0 μm wide and 2.2 μm long and motile by means of one polar flagellum. Cells are catalase and oxidase positive, strictly aerobic and non-fermentative. After 16 h incubation at 30 °C on LB agar, colonies are circular, beige in colour, flat, with regular edges and approximately 3–4 mm in diameter. Growth is observed in the presence of 0–6% (w/v) NaCl (optimum at 2–3%) and at 4–42 °C (optimum at 30–37 °C). The pH range is 5–9. A fluorescent pigment is produced on *Pseudomonas* agar F, but no pyocyanin is produced on *Pseudomonas* agar P. Biochemical tests and substrates utilized are given in diagnostic Table 2. The DNA G + C content of the type strain as deduced from the whole chromosome sequence is 60 mol%. Strain L48^T is entomopathogenic.

The type strain, L48^T (=CCUG 61470^T = CECT 7985^T), was isolated from *D. melanogaster* in Guadeloupe Island by Vodovar et al. [26].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2011.12.003.

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