Providencia vermicola sp. nov., isolated from infective juveniles of the entomopathogenic nematode *Steinernema thermophilum*

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In the course of isolating bacteria from infective juveniles of the entomopathogenic nematode *Steinernema thermophilum* Ganguly & Singh, 2000, three isolates were obtained (OP1^T, OP29 and VS3). On the basis of 16S rRNA gene sequence analysis and riboprint patterns, these three strains were identical to each other but distinct from the type strains of the five recognized species of the genus *Providencia*. Based on biochemical and genomic analysis and supported by the low (<35%) DNA–DNA relatedness between strain OP1^T and the type strain of its phylogenetically closest relative, *Providencia rettgeri* (99.5% 16S rRNA gene sequence similarity), strain OP1^T was considered to be sufficiently distinct from recognized *Providencia* species to warrant the description of a novel species. The name *Providencia vermicola* sp. nov. is proposed, with OP1^T (=DSM 17385^T=CIP 108829^T) as the type strain.

The enterobacterial genus *Providencia* comprises five species that have been isolated from the colon and faeces of humans (*Providencia alcalifaciens*, *Providencia rustigianii*), wounds, urinary tract and respiratory tract of humans (*Providencia stuartii*), urinary tract of humans, poultry, faeces from reptiles and other environments (*Providencia rettgeri*) and from faeces of penguins (*Providencia heimbachae*) [summarized by Penner (1991)]. *P. alcalifaciens* and *P. stuartii* are known for their pathogenic potential, causing diarrhoea and bacteraemia, respectively. The description of several of these species was initially based upon DNA–DNA reassociation experiments, which indicated their previous misclassification in other genera or affiliation to other species (Brenner *et al.*, 1978; Hickman-Brenner *et al.*, 1983; Müller *et al.*, 1986). The five species can be separated based on metabolic characteristics, such as acid production from some carbohydrates and several other standard tests (Müller *et al.*, 1986).

The type strains of *Providencia* species (Table 1) were obtained from the DSMZ. Isolation was performed as

described by Akhurst (1980): a mixture of infective juveniles (IJs) of nematodes of *Steinernema thermophilum* belonging to different age groups, isolated from different larvae of the greater wax moth (*Galleria mellonella*), were surface sterilized in 0.1% Hyamine 10× (methylbenzethonium chloride; Spectrum Chemicals & Laboratory Products) for 15–30 min. Nematodes were then washed three times in sterilized water in order to remove traces of Hyamine. The surface-sterilized IJs were suspended in Luria–Bertani (LB) broth or nutrient broth in a 1.5 ml microcentrifuge tube. Subsequently, the IJs were crushed manually by using a sterile tissue grinder. One loopful of the crushed suspension was then used to streak the indicator NBTA (5.0 g peptone, 3.0 g beef extract, 15 g agar, 0.025 g bromothymol blue, 0.04 g 2,3,5-triphenyl tetrazolium chloride; per litre of water) media plates. The plates were incubated for 24 h at 28 °C; individual colonies were purified by restreaking them onto fresh solid media. Isolates OP1^T, OP29 and VS3 were isolated independently from different nematode IJs at intervals of 2 weeks. These isolates were observed among other colony types, the strains of which were also isolated and await characterization (V. S. Somvanshi, E. Lang, S. Ganguly, J. Swiderski, A. K. Saxena and E. Stackebrandt, unpublished results).

Genomic DNA was extracted using the DNeasy tissue kit (Qiagen) following the manufacturer's instructions. The 16S rRNA gene was amplified as described by Rainey *et al.*

Abbreviation: IJs, infected juveniles.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences reported here are AM040495 (strain OP1^T), AM040492 (*P. rettgeri* DSM 4542^T), AM040491 (*P. stuartii* DSM 4539^T), AM040489 (*P. rustigianii* DSM 4541^T) and AM040490 (*P. heimbachae* DSM 3591^T).

Table 1. Differentiation of *Providencia* strains based on API 20E substrate reactions

Strains: 1, *P. vermicola* OP1^T (results for all isolates of *P. vermicola* were identical); 2, *P. rettgeri* DSM 4542^T; 3, *P. stuartii* DSM 4539^T; 4, *P. rustigianii* DSM 4541^T; 5, *P. heimbachae* DSM 3591^T; 6, *P. alcalifaciens* DSM 30120^T. All strains were positive for tryptophan deaminase, indole production (given as negative for *P. heimbachae* DSM 3591^T by Müller *et al.*, 1986), acetoin production, glucose fermentation/oxidation, catalase and O/F test. All strains were negative for oxidase, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, production of H₂S and gelatinase and sorbitol and melibiose oxidation/fermentation. +, Positive; –, negative; w, weak.

Reaction	1	2	3	4	5	6
Citrate utilization	+	+	+	+	–	+
Urease	+	+	–	–	–	–
Fermentation/utilization of:						
Mannitol	+	+	–	–	–	–
Inositol	+	+	+	–	+	–
Rhamnose	–	+	–	–	+	–
Amygdalin	–	+	–	–	–	–
Arabinose	+	–	–	–	–	–
Sucrose	–	–	–	w	–	+

(1996). PCR products were purified with the QIAquick PCR purification kit (Qiagen) and directly sequenced by using the CEQ Dye Terminator Cycle Sequencing kit. Products were separated on a CEQ 8000 Genetic Analysis System. The 16S rRNA gene sequences were aligned with corresponding sequences from the DSMZ database by using the ae2 editor (Maidak *et al.*, 1997). Evolutionary distances were calculated by the method of Jukes & Cantor (1969). A distance analysis dendrogram was reconstructed by using the neighbour-joining algorithm and bootstrap values were determined based on 500 resamplings (Felsenstein, 1993). DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), incorporating the modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV/VIS-spectrophotometer (Varian) equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian).

Automated ribotyping was carried out with the RiboPrinter microbial characterization system (Qualicon; DuPont). Riboprint analysis, using *EcoRI*, followed the methods described by Bruce (1996).

Physiological and biochemical tests on the three isolates and the type strains of *Providencia* species were performed at 37 °C using API 20E, API 50CH strips (bioMérieux) and Biolog GN microplates. API 20E and API 50CH strips were used according to the manufacturer’s instructions. Acid production from carbohydrates was tested on API 50CH

strips with bioMérieux medium E. Biolog GN plates (Oxoid) were incubated for 24 h before readings were taken. Wells showing a photometric value above 25 or above 30 % of the highest value of an individual strain were scored as weak or positive, respectively. Conventional biochemical tests were performed according to standard methods (Smibert & Krieg, 1994). Cultural characteristics such as colony size, shape and colour were determined after 24 h incubation at 37 °C on trypticase soy agar (Merck) plates.

The almost complete 16S rRNA gene sequence of isolate OP1^T (1517 nt) was identical to that of strains OP29 and VS3. Highest sequence similarity values were found with members of the family *Enterobacteriaceae*, specifically with members of the genus *Providencia* (>98·1 %). The phylogenetically closest type strain was *P. rettgeri* DSM 4542^T (99·5 % 16S rRNA gene sequence similarity). Except for the branching of strain OP1^T and *P. rettgeri* DSM 4542^T (99 %), bootstrap values for the other branching points were low, indicating the tentative character of the topology of the tree (Fig. 1).

Heterogeneity of the *rrn* operon, determined by the ribo-printing method with the restriction enzyme *EcoRI* (Fig. 2), indicated an identical riboprint pattern for the three isolates, thus confirming their high genomic similarity. This pattern was unique among those obtained for the type strains of *Providencia* species, which show <64 % 16S rRNA gene pattern similarity among themselves. Pattern similarity values for strains OP1^T, OP29 and VS3 and the type strains of recognized *Providencia* species are lower than 27 %. Riboprint similarities above 92 % are interpreted as indicating membership to the same ribogroup, i.e. to a taxon at the subspecific level (Anonymous, 1998). Similarity for the three new isolates was as high as 98 %. The presence of identical ribopatterns, together with identical 16S rRNA gene sequences and patterns of phenotypic characterization, for the three isolates indicates that they are descendants from the same clone recovered at different times from a mixture of IJs. These isolates are therefore considered to be members of the same species.

Fig. 1. 16S rRNA gene sequence dendrogram (De Soete, 1983) showing the position of strain OP1^T among recognized *Providencia* species. *Moellerella wisconsensis* DSM 5076^T served as a root. Bar, 1 inferred nucleotide substitution per 100 nucleotides.

Pearson correlation (Opt: 1.20 %) [0.0–100.0 %]

Fig. 2. Diversity of normalized *EcoRI* ribotype patterns found in type strains of the genus *Providencia*.

In order to investigate the physiological properties, Biolog GN and API tests were performed on strain OP1^T and the type strains of recognized *Providencia* species (Tables 1, 2 and 3). All physiological characteristics of the new isolates were identical except for the utilization of four substrates for which a variable reaction was recorded (Table 3).

Strain OP1^T is a Gram-negative, straight rod-shaped enterobacterium. It was positive for catalase and negative for oxidase, was able to ferment D-glucose and reduced nitrate.

Table 2. Differentiation of *Providencia* type strains based on API 50CHE substrate reactions

Strains: 1, *P. vermicola* OP1^T (results for all isolates of *P. vermicola* were identical); 2, *P. rettgeri* DSM 4542^T; 3, *P. stuartii* DSM 4539^T; 4, *P. rustigianii* DSM 4541^T; 5, *P. heimbachae* DSM 3591^T; 6, *P. alcalifaciens* DSM 30120^T. All strains were positive for utilization of ribose, glucose, fructose, mannose, *N*-acetylglucosamine and gluconate and negative for all other substrates provided with the API 50CH test panels except those listed below. +, Positive; –, negative; w, weak.

Acidification of:	1	2	3	4	5	6
Glycerol	+	+	+	w	+	+
Erythritol	+	+	–	–	–	–
L-Arabinose	+	–	–	–	–	–
Adonitol	+	+	–	–	+	+
Galactose	+	+	+	+	+	–
Rhamnose	–	+	–	–	+	–
Inositol	+	–	+	–	–	–
Mannitol	+	+	–	–	+	–
Sorbitol	–	–	+	–	–	–
Arbutin	–	+	–	–	–	–
Salicin	–	+	–	–	–	–
Cellobiose	–	–	+	–	–	–
Xylitol	–	–	+	–	–	–
Lyxose	–	–	+	–	–	–
D-Arabitol	+	+	–	–	+	–
L-Arabitol	+	+	–	–	–	w
2-Ketogluconate	+	–	–	–	–	–

It produced acid from L-arabinose and 2-ketogluconate and utilized L-erythritol, D-glucosaminic acid and D-glucuronic acid, all of which were negative in the other *Providencia* species. The results for the physiochemical reactions for *Providencia* species that we obtained using the API and Biolog systems were in agreement with those obtained by traditional methods (Müller *et al.*, 1986). Consistent with the description of other *Providencia* strains (Polster & Svobodova, 1964), strain OP1^T produced a brownish pigment and developed a characteristic smell on agar containing amino acids.

Previous DNA–DNA reassociation values generated under optimal hybridization conditions for the type strains of *Providencia* species ranged between 22 and 49 % (Hickman-Brenner *et al.*, 1983; Müller *et al.*, 1986). The corresponding 16S rRNA gene sequence similarity values are above 98 %. The level of DNA–DNA relatedness between strain OP1^T and the type strain of its closest phylogenetic relative, *P. rettgeri* DSM 4542^T, was 30 % (29 and 31 % similarity in two measurements using 2 × SSC buffer at 66 °C).

The combination of phylogenetic, genomic and metabolic properties suggests that isolates OP29, VS3 and OP1^T should be included in the same species and that this represents a novel species within the genus *Providencia*. Several metabolic properties, determined by two independent API and Biolog test series, were found that are discriminatory and allow identification of the novel species. The name *Providencia vermicola* sp. nov. is proposed.

Description of *Providencia vermicola* sp. nov.

Providencia vermicola (ver.mi'co.la. L. n. *vermis* worm; L. suff. *-cola* from L. n. *incola* inhabitant, dweller; N.L. n. *vermicola* inhabitant of worms).

Cells are Gram-negative rods, 2.14–5.0 × 0.57–0.71 µm. Colonies are circular, 1.8–2.2 mm in diameter, shining, slimy, convex, opaque with a brownish centre and hyaline periphery. A soluble brown pigment is produced, colouring the medium around the colonies. Colonies on MacConkey agar have a pink colour and on NBTa have a cream colour. Colonies are smooth with entire edges. An intense

Table 3. Differentiation of *Providencia* type strains based on Biolog GN substrate reactions

Strains: 1, *P. vermicola* ($n=3$); 2, *P. rettgeri* DSM 4542^T; 3, *P. stuartii* DSM 4539^T; 4, *P. rustigianii* DSM 4541^T; 5, *P. heimbachae* DSM 3591^T; 6, *P. alcalifaciens* DSM 30120^T. All strains were positive for *N*-acetyl-D-glucosamine, methylpyruvate, D-gluconic acid, succinic acid, bromosuccinic acid, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline and urocanic acid. All strains were negative for the other substrates provided with the Biolog GN test panel, except those listed below. ++, >70 % of highest reading score; +, 30–69 %; w, 25–30 %; –, <25 %; v, variable between strains.

Carbon source	1*	2	3	4	5	6
Dextrin	–	–	–	w	–	–
Tween 40	–	–	++	–	–	–
Tween 80	+	+	++	–	w	+
<i>N</i> -Acetyl-D-galactosamine	–	++	++	–	+	–
L-Arabinose	+	–	–	–	–	–
D-Arabitol	+	+	–	+	–	–
L-Erythritol	++	–	–	–	–	–
D-Fructose	++	w	+	++	++	+
D-Galactose	+	+	+	+	++	–
α -D-Glucose	+	w	+	++	++	+
<i>myo</i> -Inositol	++	+	+	+	–	–
D-Mannitol	+	+	–	+	–	–
D-Mannose	+	w	+	++	++	+
Methyl β -D-glucoside	–	w	–	–	–	–
D-Psicose	+	–	–	+	–	–
L-Rhamnose	–	+	–	+	–	–
D-Trehalose	–	–	+	–	–	–
Xylitol	+	–	+	–	–	–
Monomethyl succinate	v (–)	–	–	–	+	+
Acetic acid	+	w	w	–	+	+
<i>cis</i> -Aconitic acid	++	++	+	–	+	++
Citric acid	++	++	+	–	+	++
Formic acid	v (+)	–	–	–	+	–
D-Glucosaminic acid	+	–	–	–	–	–
Adonitol	++	+	–	+	–	+
D-Glucuronic acid	++	–	–	–	–	–
<i>p</i> -Hydroxyphenylacetic acid	+	+	–	–	+	+
α -Ketoglutaric acid	+	–	+	–	++	+
DL-Lactic acid	v (+)	–	–	w	–	–
D-Saccharic acid	–	–	+	+	–	–
Glucuronamide	v (–)	–	–	–	–	–
D-Alanine	+	–	+	–	–	–
L-Alanine	+	–	+	–	–	–
L-Alanyl glycine	++	+	+	–	–	–
Glycyl-L-aspartic acid	++	+	–	–	+	+
Glycyl-L-glutamic acid	+	+	+	w	+	+
L-Histidine	++	+	+	w	+	+
Hydroxy-L-proline	++	++	+	–	+	+
L-Leucine	–	–	–	–	w	–
L-Pyroglutamic acid	++	++	+	–	+	w
D-Serine	++	+	–	+	–	–
L-Serine	++	++	+	+	+	w
L-Threonine	w	–	–	–	w	–
DL-Carnitine	–	–	w	–	–	–
Inosine	++	++	+	–	++	–
Uridine	++	++	++	+	++	w
Thymidine	+	+	+	–	++	w

Table 3. cont.

Carbon source	1*	2	3	4	5	6
Phenylethylamine	—	—	+	—	—	—
Glycerol	++	++	+	—	+	+
α -DL-Glycerol phosphate	+	+	+	W	+	—
Glucose 1-phosphate	++	++	+	++	++	W
Glucose 6-phosphate	++	++	+	—	+	W

*In cases where the results for *P. vermicola* were variable, results for the type strain are given in parentheses.

characteristic smell is produced with growth on nutrient agar and trypticase soy agar. Negative for oxidase, positive for catalase, urease and nitrate reduction and able to ferment D-glucose. Growth occurs up to 41 °C. Differential biochemical characteristics include acid production from L-arabinose and 2-ketogluconate and utilization of L-erythritol, D-glucosaminic acid and D-glucuronic acid.

The type strain, OP1^T (=DSM 17385^T=CIP 108829^T), was isolated from the crushed IJs of the nematode *Steinernema thermophilum* Ganguly & Singh, 2000, collected in soils of the Indian Agricultural Research Institute, New Delhi, India.

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