Providencia sneebia sp. nov. and *Providencia burhodogranariea* sp. nov., isolated from wild *Drosophila melanogaster*

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Multiple isolates of the genus *Providencia* were obtained from the haemolymph of wild-caught *Drosophila melanogaster* fruit flies. Sixteen isolates were distinguished from the six previously described species based on 16S rRNA gene sequences. These isolates belonged to two distinct groups, which we propose each comprise previously undescribed species. Two isolates, designated A^T and B^T, were characterized by DNA sequences of the *fusA*, *lepA*, *leuS*, *gyrB* and *ileS* housekeeping genes, whole-genome DNA–DNA hybridizations with their nearest relatives and utilization of substrates for metabolism. The closest phylogenetic relatives of strain A^T are strain B^T (86.9 % identity for the housekeeping genes) and *Providencia stuartii* DSM 4539^T (86.0 % identity). The closest phylogenetic relatives of strain B^T are strain A^T (86.9 % identity) and *P. stuartii* DSM 4539^T (86.6 % identity). The type strains of described species in this genus shared between 84.1 and 90.1 % identity for these sequences. DNA–DNA hybridization between the strain pairs A^T–B^T, A^T–*P. stuartii* DSM 4539^T and B^T–*P. stuartii* DSM 4539^T all resulted in less than 25 % relatedness. In addition, patterns of utilization of amygdalin, arbutin, aesculin, salicin, D-sorbitol, trehalose, inositol, D-adonitol and D-galactose distinguish strains A^T and B^T from other members of this genus. Strains A^T and B^T therefore represent novel species, for which the names *Providencia sneebia* sp. nov. (type strain A^T =DSM 19967^T =ATCC BAA-1589^T) and *Providencia burhodogranariea* sp. nov. (type strain B^T =DSM 19968^T =ATCC BAA-1590^T) are proposed.

The genus *Providencia*, in the family *Enterobacteriaceae*, currently has six recognized species. Members of the genus have repeatedly been found in association with humans, insects and many other vertebrate and invertebrate animals in both pathogenic and non-pathogenic contexts (Penner & Hennessy, 1979; Müller *et al.*, 1986; Yoh *et al.*, 2005; Somvanshi *et al.*, 2006). We describe here two novel *Providencia* species isolated from the haemolymph of field-captured *Drosophila melanogaster* fruit flies.

D. melanogaster flies were collected in State College, PA, USA, in 1998 and 2001. Individual flies were surface-sterilized by UV irradiation prior to haemolymph extraction with pulled-glass microcapillary needles. The haemolymph was used to inoculate 1 ml liquid cultures of brain heart infusion (BHI). Liquid cultures were grown aerobically for 24 h at 37 °C. Enriched cultures were then streaked on BHI agar plates and individual colonies were selected for identification. Seventeen of a total of 337 *D. melanogaster* flies yielded bacterial isolates assignable to the genus *Providencia* based on partial sequences of the 16S rRNA gene, amplified using primers fd1 and rp2 as described by Weisburg *et al.* (1991). These isolates clustered into four groups based on partial 16S rRNA gene sequences (978 nt), termed A, B, C and D (Supplementary Fig. S1, available in IJSEM Online). The sole isolate belonging to group C was subsequently identified as *Providencia rettgeri* based on its metabolic profile and DNA sequence at housekeeping genes (described below). The remaining isolates did not match described species closely. Isolates A^T, A75, A91, A101, A102, B^T, B18, B97, D and D37 were chosen for further characterization. These isolates were compared with the type strains *Providencia stuartii* DSM 4539^T, *P. alcalifaciens* DSM 30120^T, *P. heimbachae* DSM 3591^T, *P. rustigianii* DSM 4541^T, *P. rettgeri* DSM 4542^T and *P. vermicola* DSM 17385^T, all obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany).

The GenBank/EMBL/DDBJ accession numbers for the sequences of the 16S rRNA, *ileS*, *gyrB*, *fusA*, *lepA* and *leuS* genes of the type strains of six described *Providencia* species and the novel strains are EU587018–EU587118, as detailed in Supplementary Table S2.

Details of primers used for PCR amplification of housekeeping genes and sequence accession numbers and a 16S rRNA gene sequence-based neighbour-joining tree are available as supplementary material with the online version of this paper.

Metabolic characteristics were determined from single colonies grown for 24 h at 37 °C on Luria–Bertani (LB) agar plates. Metabolic profiles were determined with API 20E and API 50CH test strips (bioMérieux). Inocula for API 20E strips were prepared in distilled water and inocula for API 50CH strips were prepared using the API 50 CHB/E medium. Assays were interpreted after 30 h incubation at 25 °C. Test strips were run at least twice for strains A^T and B^T and all of the reference type strains except *P. rustigianii* DSM 4541^T. Test strips were run once for *P. rustigianii* DSM 4541^T and non-type strains of the novel species.

The metabolic profiles of the novel isolates were distinct from each other and from all described *Providencia* species (Table 1). Results for previously described species varied slightly from published reports by Somvanshi *et al.* (2006), Farmer *et al.* (1985) and Hickman-Brenner *et al.* (1983). These deviations may be due to differences in the test method, temperatures and reaction durations employed by different authors. Isolates A^T, A75, A91, A101 and A102 all had identical metabolic profiles. Isolates B^T, B18, B97, D and D37 had identical metabolic profiles except for utilization of amygdalin, rhamnose and D-sorbitol, for

Table 1. Differentiation of *Providencia* strains based on metabolic substrate reactions

Strains: 1, *P. sneebia* sp. nov. strains A^T, A75, A91, A101 and A102; 2, *P. burhodogranariea* sp. nov. strains B^T, B18 and B97; 3, *P. burhodogranariea* sp. nov. strains D and D37; 4, *P. vermicola* DSM 17385^T; 5, *P. rettgeri* DSM 4542^T; 6, *P. stuartii* DSM 4539^T; 7, *P. rustigianii* DSM 4541^T; 8, *P. heimbachae* DSM 3591^T; 9, *P. alcalifaciens* DSM 30120^T. All strains were tested under aerobic conditions. Isolates of *P. sneebia* sp. nov. and *P. burhodogranariea* sp. nov. yielded virtually identical results (see text). Each strain was tested at least twice, except *P. rustigianii* DSM 4541^T and reference strains of the novel species, which were each tested once. All strains were positive for tryptophan deaminase[a]† and oxidation/fermentation of D-glucose[ab], glycerol[b], D-ribose[b], D-fructose[b], D-mannose[b], *N*-acetylglucosamine[b] and potassium gluconate[b]. All strains were negative for β-galactosidase[a], arginine dihydrolase[a], lysine decarboxylase[a], ornithine decarboxylase[a], H_2S production[a], acetoin production[a] (given as positive for all strains by Somvanshi *et al.*, 2006), gelatinase[a] and oxidation/fermentation of melibiose[ab], D- and L-arabinose[b], L-xylose[b], methyl β-D-xylopyranoside[b], L-sorbose[b], dulcitol[b], methyl α-D-mannopyranoside[b], methyl α-D-glucopyranoside[b], amygdalin[b], cellobiose[b], lactose[b], sucrose[b], inulin[b], melezitose[b], raffinose[b], starch[b], glycogen[b], gentiobiose[b], turanose[b], D-tagatose[b], D- and L-fucose[b] and potassium 5-ketogluconate[b]. Test strips did not give repeatable results within isolates for citrate utilization[a] and indole production[a], so results from these assays are not presented. +, Positive; –, negative; V, variable between strains; ?, variable within isolate; W, weak.

Characteristic	1	2	3	4	5	6	7	8	9
Urease	+	–	–	?[z]*	+	–	–	–	–
Utilization of:									
L-Arabinose[a]†	–	+	+	+	W[z]	–	W[z]	–	–
D-Adonitol[b]	–	+	+	+	+	–	–	+	+
Amygdalin[a]	+	–	V	–	+	–	–	–	–
D-Arabitol[b]	+	+	+	+	+	–	–	+	–
L-Arabitol[b]	–	–	–	+	+	–	–	+[z]	–
Arbutin[b]	+	–	–	–	+	–	–	–	–
Erythritol[b]	–	–	–	+	?[z]	–	–	+[z]	–
Aesculin[b]	+	–	–	–	+[z]	–	–	–	–
D-Galactose[b]	–	–	–	+	+	+	+	+	–
Inositol[a]	–	+	+	+	+	W	–	+	–
Inositol[b]	–	+	+	+	+[z]	+	–	+	–
2-Ketogluconate[b]	–	W	+	+	+[z]	–	–	+[z]	–
D-Lyxose[b]	–	–	–	–[z]	–[z]	+[z]	–	–[z]	–
Maltose[b]	–	–	–	–	–	–	–	+[z]	–
D-Mannitol[a]	+	+	+	+	+	–	–	–	–
D-Mannitol[b]	+	+	+	+	+	–	–	–[z]	–
L-Rhamnose[a]	–	–	V	–	–	+	–	–	+
L-Rhamnose[b]	–	–	–	–	+	–	–	+	–
Salicin[b]	+	–	–	–	+	–	–	–	–
D-Sorbitol[a]	+	V	V	–	–	–	–	–	–
D-Sorbitol[b]	+	–	–	–	–	–	–	–	–
Sucrose[a]	–	?	?	–	–	–	–[yz]	–	–[z]
Trehalose[b]	+	+	+	–	–	–[xy]	–	–	–
Xylitol[b]	–	–	–	–	–	+	–	–	–
D-Xylose[b]	W	–	–	–	–	–	–	–	–

*Results differ from those published previously by: *x*, Hickman-Brenner *et al.* (1983); *y*, Farmer *et al.* (1985); *z*, Somvanshi *et al.* (2006).
†Results were determined using the API 20 E (*a*) or API 50 CH (*b*) test strip.

which there was variation among isolates (Table 1). The *Providencia* species represented by A^T is uniquely able to utilize D-sorbitol and D-xylose. In contrast to all previously described species of *Providencia*, isolates A^T and B^T are able to utilize trehalose.

Partial sequences of five housekeeping genes (*fusA*, 616 nt; *lepA*, 735 nt; *leuS*, 412 nt; *gyrB*, 817 nt; *ileS*, 920 nt) were obtained from ten novel isolates and from the six reference *Providencia* type strains. Housekeeping genes were amplified and sequenced using a combination of degenerate primers described by Santos & Ochman (2004) and *Providencia*-specific custom primers (Supplementary Table S1). PCR products were prepared for sequencing by 1 h incubation with exonuclease I and shrimp alkaline phosphatase (both from USB Corporation) and sequenced using ABI BigDye Terminator chemistry on an Applied Biosystems Automated 3730 DNA Analyzer. In some cases, amplicons were purified by agarose gel electrophoresis prior to sequencing. Sequences were aligned using CodonCode Aligner (CodonCode Corporation). GenBank/EMBL/DDBJ accession numbers of the new sequences are detailed in Supplementary Table S2. Phylogenetic analysis was performed using MEGA version 3.1 (Kumar *et al.*, 2004) both on alignments of individual genes and on an alignment of a concatenation of all six genes. Distances were calculated based on Jukes–Cantor-corrected percentage divergence and clustering was performed by neighbour-joining. Bootstrap values from 1000 replications were used to assess confidence at each node.

The percentage divergence among type strains of described *Providencia* species across the concatenated housekeeping genes ranged from 9.9 % between *P. alcalifaciens* DSM 30120^T and *P. rustigianii* DSM 4541^T to 15.9 % between *P. stuartii* DSM 4539^T and *P. heimbachae* DSM 3591^T (Fig. 1). Within group A of the novel isolates, the maximum observed divergence was 0.3 %. Based on this level of sequence similarity, strains A^T, A75, A91, A101 and A102 are considered to belong to the same species. Within groups B and D of the isolates, the maximum observed divergence was 0.2 %. Isolates of groups B and D differed by only 6.3 %, lower than the minimum divergence observed between type strains of described *Providencia* species. Based on their sequence similarity and nearly identical metabolic profiles, strains B^T, B18, B97, D and D37 are considered to belong to the same species. Strain B^T differed from strain A^T by 13.1 % and from *P. stuartii* DSM 4539^T by 13.4 %. Strain A^T differed from *P. stuartii* DSM 4539^T by 14.0 %. These percentage divergence values fall well above the minimum percentage divergence observed between type strains of described *Providencia* species, supporting the hypothesis that strains A^T and B^T represent distinct and novel *Providencia* species.

DNA–DNA hybridizations were performed between all pairwise combinations of strains A^T and B^T and *P. stuartii* DSM 4539^T, which was inferred to be the nearest described relative based on DNA sequences at the housekeeping genes. DNA isolations and DNA–DNA hybridizations were performed by the DSMZ following the methods described by Cashion *et al.* (1977), De Ley *et al.* (1970) and Huß *et al.* (1983). The reassociation value for the strain pair A^T–*P. stuartii* DSM 4539^T was 13.0 % (mean of 12.7 and 13.3 %, obtained from two separate measurements). The reassociation value for the strain pair B^T–*P. stuartii* DSM 4539^T was 18.8 % (mean of 13.8 and 23.8 %, obtained from two separate measurements). The reassociation value for the strain pair A^T–B^T was 13.1 % (mean of 7.6 and 18.6 %, obtained from two separate measurements). Previous studies have reported reassociation values that range from 22 to 49 % between strains of separate species in this genus (Hickman-Brenner *et al.*, 1983; Müller *et al.*, 1986; Somvanshi *et al.*, 2006). The results from the DNA–DNA hybridizations fall well below the 70 % reassociation threshold recommendation of Wayne *et al.* (1987) for designation of a novel species. These results indicate that strains A^T and B^T are significantly distinct from each other and from their nearest described relative in *Providencia*.

The results from the housekeeping gene sequencing, DNA–DNA hybridization and metabolic analysis meet the

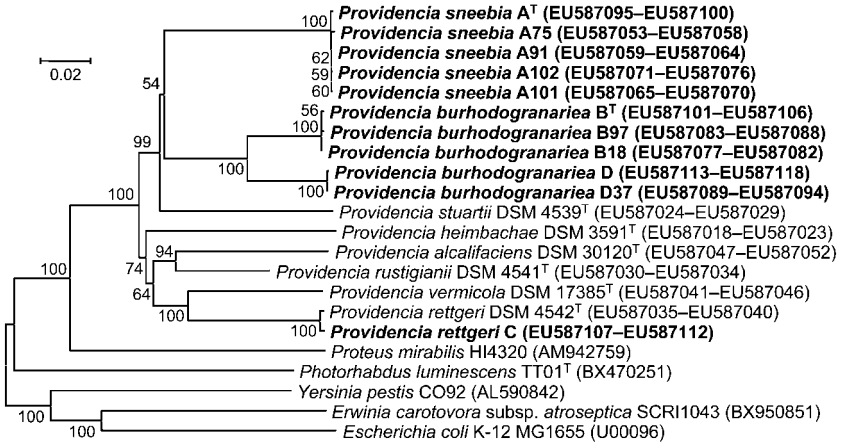

Fig. 1. Neighbour-joining phylogenetic tree based on concatenated sequences from the 16S rRNA gene, *fusA*, *lepA*, *leuS*, *gyrB* and *ileS* loci (4478 nt) showing the positions of the novel strains within *Providencia*. Sequences for strains of *Proteus mirabilis*, *Photorhabdus luminescens*, *Yersinia pestis*, *Erwinia carotovora* and *Escherichia coli* were obtained from GenBank (accession numbers in parentheses). Bootstrap values on each node are percentages based on 1000 replicates. Bar, 2 % sequence divergence.

requirements outlined by Wayne *et al.* (1987) for designating a bacterial species as novel. Based on these results, strains A^T, A75, A91, A101 and A102 belong to a single novel species, for which the name *Providencia sneebia* sp. nov. is proposed. Likewise, strains B^T, B18, B97, D and D37 belong to a second novel species, for which the name *Providencia burhodogranariea* sp. nov. is proposed.

Description of *Providencia sneebia* sp. nov.

Providencia sneebia (snee′bi.a. N.L. fem. adj. *sneebia* of University SNEEB, a series of informal academic gatherings at Cornell University where properties of these bacteria were extensively discussed).

Gram-negative, rod-shaped bacterium. Colonies grown on LB agar for 48 h at 37 °C are up to 4 mm in diameter, white, opaque, glossy and convex. Growth occurs faster at 37 °C than at 25 °C. Unique among currently known members of the genus *Providencia* in being able to produce acid from amygdalin, arbutin, aesculin, salicin, D-xylose, D-sorbitol and trehalose but not from inositol, D-adonitol or D-galactose.

The type strain is A^T (=ATCC BAA-1589^T =DSM 19967^T). This and a number of reference strains were isolated from *Drosophila melanogaster* captured in an apple orchard in State College, PA, USA.

Description of *Providencia burhodogranariea* sp. nov.

Providencia burhodogranariea (bu.rho.do.gran.ar′ie.a. Gr. pref. *bu-* big; Gr. pref. *rhodo-* red; L. n. *granaria* a barn; N.L. fem. adj. *burhodogranariea* of the Big Red Barn, the name of the building where academic discussions of these bacteria were held).

Gram-negative, rod-shaped bacterium. Colonies grown on LB agar for 48 h at 37 °C are up to 4 mm in diameter, white, opaque, glossy and convex. Growth occurs faster at 37 °C than at 25 °C. After 24–48 h of growth, colonies express brown pigmentation in their centres. Unique among currently known members of the genus *Providencia* in being able to produce acid from D-adonitol, trehalose and inositol but not from D-galactose.

The type strain is B^T (=ATCC BAA-1590^T =DSM 19968^T). This and a number of reference strains were isolated from *Drosophila melanogaster* captured in an apple orchard in State College, PA, USA.

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