Verminephrobacter eiseniae gen. nov., sp. nov., a nephridial symbiont of the earthworm *Eisenia foetida* (Savigny)

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A Gram-negative, flagellated, heterotrophic, catalase-negative, rod-shaped bacterium previously identified as an earthworm symbiont was isolated from nephridia of the earthworm *Eisenia foetida*. Comparisons of 16S rRNA gene sequences indicated its relatedness to the betaproteobacterial genus *Acidovorax* and the novel isolates shared 92–94 % sequence similarity with recognized species of this genus. Gene sequence phylogenies revealed that the group of earthworm symbionts formed a cohesive and independent clade. The DNA G + C content was 67.0 ± 0.2 mol%. Major fatty acids were C_{16:0}, C_{16:1 ω 7c} and C_{17:0} cyclo. While capable of growing in fully aerated media, all isolates favoured low oxygen concentrations and all required biotin or a mix of amino acids in order to grow on defined mineral media. Based on phylogenies inferred from three housekeeping gene sequences (*gap*, *recA* and *rpoC*), DNA–DNA hybridization values, the unique ecology and the distinct physiology of the novel strains, the new genus *Verminephrobacter* gen. nov. is proposed for the earthworm nephridial symbionts. The name *Verminephrobacter eiseniae* sp. nov. is proposed for the type species with strain EF01-2^T (=ATCC BAA-1489^T=DSM 19286^T) as the type strain of the type species.

The presence of rod-shaped bacteria in the nephridia (excretory organs) of several species of lumbricid earthworms (Annelida: Oligochaeta: Crassidellata Jamieson, 1988; Lumbricidae Claus 1876) was initially reported in light microscopy studies by Knop (1926). Subsequent

studies identified the dominant bacteria, localized predominantly in the ampullum (the medial region of the nephridial second loop), as Gram-negative organisms (Scott & Musgrave, 1971) closely related to the betaproteobacterial genus *Acidovorax* (Schramm *et al.*, 2003). Unique 16S rRNA betaproteobacterial ribotypes have been recovered from different earthworm species. The transmission of the symbiont through its deposition in the egg capsule has been recently demonstrated in the earthworm *Eisenia foetida* Savigny by Davidson & Stahl (2006).

Acidovorax-like 16S rRNA gene sequences are frequently recovered from wastewater treatment plants and other aquatic environments (Schweitzer *et al.*, 2001; Hoshino *et al.*, 2005; Manefield *et al.*, 2005). The genus *Acidovorax* includes species isolated from soil and sludge (Willems *et al.*, 1990; Schulze *et al.*, 1999; Heylen *et al.*, 2008), in addition to a group of plant pathogens with a varied host range (Willems *et al.*, 1992; Gardan *et al.*, 2000, 2003). The present study reports the isolation and characterization of a relative of the genus *Acidovorax* from the nephridia of *E. foetida*. This is the first representative of a group affiliated with the genus that forms a recognizably stable association with animal hosts. Based on the unique ecology of this organism relative to other known species of the genus *Acidovorax* and its distinct characteristics reported herein, we propose the designation of the genus

Abbreviations: ACM, *Acidovorax* complex medium; ACMP, ACM Phytigel; CFA, cyclic fatty acids; CIA, chloroform-isoamyl alcohol; FAME, fatty acid methyl esters; FISH, fluorescence *in situ* hybridization; ML, maximum-likelihood; MLST, multilocus sequence typing; MP, maximum-parsimony; NJ, neighbour-joining; PCIA, phenol/chloroform-isoamyl alcohol; PFGE, pulsed field gel electrophoresis; PHB, polyhydroxybutyrate; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Verminephrobacter eiseniae* isolates EF01-1, EF01-2^T, EF02-2, EF03-1, EF03-2, EF04-1, EF05-1 and EF05-2 are DQ327662, DQ327663, DQ327664, DQ327665, DQ327666, DQ327667, DQ327668 and DQ327669, respectively. The GenBank accession numbers for the MLST sequences are EU165058–88 and EU350960.

Details of the methods used for soil DNA surveys and siderophore production and uptake are available as supplementary methods with the online version of this paper. Also available are supplementary tables giving the results of DNA–DNA hybridization studies and the pairwise sequence identities of 16S rRNA sequences. Supplementary figures show additional phylogenetic trees, the reactions in 'deep phytigel' tubes, vitamin requirements and siderophore production assays for the strains studied.

Verminephrobacter gen. nov. to include betaproteobacterial denizens of lumbricid earthworm nephridia. The isolates recovered from *E. foetida* are designated as representing the type species for the new genus and the name *Verminephrobacter eiseniae* sp. nov. is proposed.

Cultures were regularly initiated from frozen stocks and were maintained in *Acidovorax* complex medium [ACM; containing 1^{-1} : 0.5 g yeast extract (Difco-Becton Dickinson), 1.0 g Casamino acids (Difco), 2.0 g pyruvic acid, 2.0 g L-glutamine, 0.3 g KH_2PO_4 , 0.3 g MgSO_4 and 2.0 g MOPS, with the pH adjusted to 7.2–7.3 with 5 M KOH before autoclaving]. The following gelling agents were tested: Bacto agar (Difco), Noble agar (Sigma Aldrich), agarose (JT Baker–Mallinckrodt Baker) and Phytigel (Sigma Aldrich). Only Phytigel afforded consistent isolation and growth of nephridial bacteria. Plates contained 1.5 % Phytigel. Basal defined media consisted of (1^{-1} distilled water): 10 ml $100\times$ mineral salts solution (unless noted otherwise, all chemicals were obtained from Sigma Aldrich; 1^{-1} : 250 g NaCl, 50 g KH_2PO_4 , 10 g MgSO_4 , 1 g CaCl_2), 1 ml SL12 trace metal solution (1^{-1} : 3 g sodium EDTA, 1.1 g FeCl_2 , 6 mg H_3BO_3 , 190 mg $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, 100 mg $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$, 70 mg ZnCl_2 , 36 mg $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$, 24 mg $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$ and 2 mg $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$) and 1 ml $1000\times$ vitamin solution (1^{-1} : 50 mg *p*-aminobenzoic acid, 20 mg folic acid, 50 mg nicotinic acid, 50 mg calcium pantothenate, 50 mg riboflavin, 50 mg thiamine hydrochloride, 100 mg pyridoxine, 50 mg thioctic acid, 5 mg cyanocobalamin and 1 mg biotin). The medium was buffered with 20 mM MOPS at pH 7.2–7.3. Carbon and nitrogen sources were added as appropriate.

Isolate EF01-2^T was designated as the type strain of the novel isolates deposited. Strain EF05-2 was also deposited as a reference strain (=DSM 19250). The following reference strains were used for comparisons: *Acidovorax anthurii* DSM 16745^T, *A. avenae* subsp. *avenae* DSM 7227^T, *A. avenae* subsp. *citruilli* AAC00-1, *A. defluvii* DSM 12644^T, *A. delafieldii* ATCC 17505^T, *A. facilis* ATCC 11228^T, *A. konjaci* DSM 7481^T, *A. temperans* ATCC 49665^T and *A. valerianellae* DSM 16619^T.

In order to isolate and quantify the nephridial bacteria, earthworms were maintained as previously described by Davidson & Stahl (2006). For dissection, specimens were anaesthetized in $1\times$ PBS with 4 % (v/v) ethanol. The body wall was separated along a dorsal, longitudinal incision and held in place with dissection pins. The intestines were removed and the body tissues were rinsed with sterile $1\times$ PBS/4 % ethanol. Nephridia (20–50) were removed with forceps, rinsed once in sterile $1\times$ PBS and pooled into a microcentrifuge tube with sterile $1\times$ PBS. Nephridia were crushed with a sterile micro pestle, diluted and plated. Plates were sealed with Parafilm and incubated at room temperature ($\sim 25^\circ\text{C}$). Colonies of the predominant morphology on the highest dilution plates presenting growth were streaked for purity and incubated as above. The resulting colonies were screened by fluorescence *in situ*

hybridization (FISH) using the *Acidovorax*-specific probe LSB145 (Schweitzer *et al.*, 2001) and then inoculated into ACM broth. Cultures were incubated at room temperature with agitation in a rotary shaker at 250 r.p.m. After 6–10 days, the identity of the isolates was confirmed by FISH and 16S rRNA gene sequencing. Similar isolation procedures were used for the isolation of symbionts from the earthworm species *Lumbricus terrestris* and *Aporrectodea longa* (N. Pinel, unpublished).

After 15–20 days incubation at room temperature, ACM-Phytigel (ACMP) plates inoculated with the 10^{-4} – 10^{-5} dilutions from nephridial suspensions presented, almost exclusively, circular, convex colonies of 0.5–2 mm in diameter, with entire or lightly erose margins and a light bisque to peach colour. The colonies showed negative Gram staining (not shown) and positive hybridizations to the LSB145 oligonucleotide probe.

Material for the determination of the number of colony forming units (c.f.u.) per nephridium was prepared as above from four sexually mature (clitellated) worms. Serial dilutions were plated on ACMP plates in triplicate or quadruplicate. Colony counts indicated a mean bacterial density of $3.3 \pm 1.9 \times 10^5$ c.f.u. per *E. foetida* nephridium.

For phylogenetic analyses, chromosomal DNA was extracted and purified by the Marmur protocol (Marmur, 1963) with the following modifications after cell lysis: the lysate was extracted once with Tris-SDS buffer-saturated phenol for 1 h (Tris-SDS buffer; 100 mM Tris/HCl pH 8.0; 50 mM EDTA pH 8.0; 100 mM NaCl; 1 % w/v SDS). The aqueous phase was then extracted with phenol/chloroform-isoamyl alcohol (PCIA; 25:24:1) and chloroform-isoamyl alcohol (CIA; 24:1). DNA was precipitated with one volume of 2-propanol, resuspended in $1\times$ TE buffer and treated with RNase A, followed by PCIA and CIA extractions, ethanol precipitation and resuspension in $1\times$ TE.

The nearly complete 16S rRNA gene was amplified and sequenced according to standard methods with primers GM3 and GM4 (Muyzer *et al.*, 1993). The resultant sequences (1455–1463 bp) for all eight isolates were aligned in ARB (Ludwig *et al.*, 2004) against the Ribosomal Database Project (RDP) database (Cole *et al.*, 2003) with manual refinement. The primer pairs gapf-1 (5'-AAYGGYTTYGGCCGCATC-3')/gapr-1 (5'-CACSCG-CACCATYTCCAG-3'), recAf-1 (5'-GGCAAGGGCACS-ATCATG-3')/recAr-1 (5'-CGTTGWAGGCGTACCAGGC-3'), and rpoCf-1 (5'-CCTGTTCAAGCAATTCACG-3')/rpoCr-1 (5'-ACCTTGTTGTAGCGCTCG-3') were used to amplify and sequence fragments from glyceraldehyde-3-phosphate dehydrogenase (type I; *gap*; ~ 859 nt), *recA* (~ 716 nt) and the DNA-directed RNA polymerase, beta' subunit (*rpoC*), respectively, unless the corresponding sequences were available from public databases. Interior sequencing of *rpoC* was achieved with primers rpoC-seqF2 (5'-AGCGCAAGGAATAYGG-3') and rpoC-seqR2 (5'-GAGCACCACGTCYTG-3'); sequences of 1290 nucleo-

tides were used. Sequence alignments were constructed with CLUSTAL W (Thompson *et al.*, 1994) and refined manually. Maximum-parsimony (MP), evolutionary distance (neighbour-joining; NJ) and maximum-likelihood (ML) phylogenetic analyses were performed in PHYLIP (Felsenstein, 1989).

Among the isolated symbionts, the 16S rRNA gene sequences were $98.5 \pm 0.4\%$ similar. The symbiont gene sequences were $94.9 \pm 0.5\%$ similar to those of nephridial clones obtained from other earthworm species, $92.5 \pm 0.5\%$ similar to those of reported plant pathogens of the genus *Acidovorax* and $93.3 \pm 0.3\%$ similar to other species within the genus. Mean 16S rRNA gene sequence similarity values among plant-pathogenic and non-symbiotic *Acidovorax* species ranged from 96.2–97.4%. The *E. foetida* isolates formed a monophyletic group within the clade of earthworm nephridial symbionts. All 16S rRNA gene phylogenies (Fig. 1a, NJ; MP and ML not shown) supported the cohesion of the novel symbiotic group, but allowed only limited resolution of the branching pattern among the sequences examined. Three well-defined groups were supported by phylogenetic reconstructions with *rpoC* or concatenated *recA/gap* gene sequences (Fig. 1b, c and see Supplementary Fig. S1 in IJSEM Online). One of the groups encompassed the earthworm symbionts, another included most of the known plant pathogenic species of the genus *Acidovorax* and a third comprised most of the remaining species. The branching order of these three groups could not be confidently established (bootstrap support above 50%) from the inferred topologies.

The DNA G+C content was determined as previously described (Mesbah *et al.*, 1989) with the following modifications: DNA was digested with 10 U mung bean nuclease and 50 U S1 nuclease. Separation of nucleosides was performed isocratically for 15 min with a flow rate of 1 ml min^{-1} on a mobile phase of 95% 20 mM triethylamine phosphate (pH 5.1)/5% methanol, through a Zorbax SB-C18 column ($4.6 \times 150 \text{ mm}$; $5.0 \mu\text{m}$ particle size; Agilent Technologies). DNA samples from *A. temperans* and *A. delafieldii* were used as controls. The DNA G+C content (determined for isolates EF01-1, EF01-2^T, EF02-2 and EF05-1) was $67.0 \pm 0.2 \text{ mol\%}$, well within the 60–72 mol% values reported for other species of the genus *Acidovorax* (Schulze *et al.*, 1999; Gardan *et al.*, 2000, 2003), and in close agreement with the 65.2 mol% observed for the genome sequence of isolate EF01-2 (unpublished observations).

DNA–DNA hybridizations were conducted with the microtitre plate technique (Ezaki *et al.*, 1989) as described by Willems *et al.*, (2001), and modified as follows: before labelling with photobiotin, probe DNA was fragmented through nebulizers (Invitrogen) under a stream of N_2 at a pressure of 28 p.s.i. for 1.5 min, yielding a final fragment range of 250–2000 kb. Probe DNA was labelled under direct sunlight for 1 h on ice. The hybridization temperature was 50 °C. The chromogenic substrate 2-nitrophenyl-

β -galactopyranoside (ONPG) replaced the fluorogenic substrate 4-methylumbelliferone used in the original protocol. After a 30 min incubation at 37 °C, the chromogenic reactions were developed for at least 15 min with $10 \mu\text{l}$ 2 M sodium carbonate. Hybridization values were calculated from the percent absorbance at 405 nm, with the homologous reaction representing 100%. Salmon DNA was immobilized as a background absorbance control. All hybridizations were run in triplicate wells on at least three plates. The mean standard deviation of the method was $\pm 7.6\%$. The complete results of the DNA–DNA hybridization experiments are provided in Supplementary Table S1 (see IJSEM Online). The mean DNA–DNA hybridization values were $99 \pm 2\%$ (range 94–107%) among all eight isolated symbionts. In contrast, mean reassociation values to type species of the genus *Acidovorax* ranged from 8 to 27%. The DNA–DNA hybridization results supported the status of the isolated symbionts as representing a novel species. While Willems *et al.* (2001) used the reciprocal hybridizations as internal controls, we found that in some cases reciprocal hybridization gave consistently disparate values, possibly reflecting differences in genome sizes among the species included in the assays. The discrepancies did not alter the main conclusion from our results.

In order to examine the DNA molecules of the isolated strains, pulsed field gel electrophoresis (PFGE) of genomic DNA was performed on a CHEF-DR III system (Bio-Rad Laboratories). DNA plugs were prepared as per the protocol given in the instrument documentation, with $\sim 5.0 \times 10^8$ cells per agarose plug.

For detection of large extrachromosomal molecules, DNA plugs were treated with S1 nuclease as previously described (Barton *et al.*, 1995) and examined using PFGE. The alkaline lysis miniprep protocol was used for extracting small plasmids. An extrachromosomal DNA molecule was detected in isolates EF01-1, EF01-2^T, EF02-2 and EF04-1 (data not shown), which migrated as a 98 kb molecule from untreated DNA plugs and as a 35 kb molecule after S1-linearization (not shown). By sequencing the genome of isolate EF01-2, this molecule was confirmed as a circular, 31.2 kb plasmid (unpublished observations). Four extrachromosomal molecules were observed in *A. temperans* DNA preparations, with migration rates suggestive of 375, 155, 96 and 47 kb before, or 212, 155, 55 and 29 kb after S1 linearization, respectively (not shown). Additional extrachromosomal molecules were detected through PFGE for *A. defluvii* (212 and 169 kb) and *A. facilis* (193 kb), but only after S1 linearization. A 2.3 kb plasmid was isolated from *A. defluvii* (not shown).

For cellular fatty acid analysis, fatty acid methyl esters (FAME) were extracted and chromatographed by the Microbial Identification Company (MIDI; Microbial ID; Smibert & Krieg, 1994) according to standard protocols. Cultures of isolated symbionts and the type strains of *A. avenae* subsp. *avenae*, *A. facilis*, *A. konjaci* and *A. temperans*

Fig. 1. Neighbour-joining phylogenetic reconstructions from (a) nearly complete 16S rRNA gene sequences; (b) concatenated partial *recA/gap* nucleotide sequences; (c) partial *rpoC* nucleotide sequences. Bootstrap values were derived from 1000 replicates. Nodes with <50 % bootstrap support were eliminated. Entries in bold type correspond to isolates described in this report. *Organisms associated with earthworm nephridia; **reported plant-pathogenic species of the genus *Acidovorax*. *Bordetella bronchiseptica* RB50 served as an outgroup in all reconstructions. Trees constructed with maximum-parsimony and maximum-likelihood algorithms are available as supplementary figs in IJSEM Online. 'Acidovorax sp.' JS42 is misnamed in current databases; it is included as it is part of a *Verminephrobacter eiseniae* sp. nov. comparative genome analysis project (unpublished data). Bars, 1 nucleotide substitution per 100 nucleotides.

(see above) were grown on ACMP plates for either 15 days (isolated symbionts) or 48 h (other strains of the genus *Acidovorax*) before biomass was collected. The differences in incubation time reflected differences in growth rate and the time required for obtaining sufficient biomass. To account for growth medium effects, results for the recognized species of the genus *Acidovorax* were compared with data from Willems *et al.* (1990, 1992). FAME peaks were identified as equivalent chain-length (ECL) and their area expressed as percentages of total peak area. Similarities

were calculated using the correlation coefficient algorithm (Bousfield *et al.*, 1983) after removal of peaks with <1 % total peak area. Cluster analyses were performed with the PC-ORD software package, employing the Sorensen similarity index and the UPGMA and NJ algorithms.

The fatty acid composition profiles for the eight symbiotic isolates had similarity values of $98 \pm 1\%$, while their similarity to the profiles from the *Acidovorax* species examined was $91 \pm 1\%$. The similarity to fatty acid profiles

reported for other species of the family *Comamonadaceae* was below 90 % (not shown; Willems *et al.*, 1990, 1992; Schulze *et al.*, 1999). The profiles obtained in this study for the type strains of the reference *Acidovorax* species growing on ACMP plates and those reported in the literature for the corresponding species differed by less than 1 %. The dominant components included peaks with ECL values for palmitic ($C_{16:0}$, 27–31 %), palmitoleic ($C_{16:1}$, 16–35 %) and *cis*-9,10-methylenehexadecanoid acid ($C_{17:0}$ cyclo, 9–26 %); the latter being exclusive to the isolated symbionts among other reference strains (Table 1). Cyclopropanes (such as $C_{17:0}$ cyclo) are derived from the corresponding unsaturated fatty acids (Grogan & Cronan, 1997), which could explain the reduction in palmitoleic acid in the novel isolates relative to the amounts found in the reference *Acidovorax* species. Differential expression of cyclic fatty acids (CFA) according to growth state has been observed for many organisms (Grogan & Cronan, 1997; Könneke & Widdel, 2003) and their appearance has been hypothesized as a strategy to preserve membrane integrity during starvation (Guckert *et al.*, 1986) as CFA are more refractory to bacterial lipases than their *cis*-monounsaturated acid precursors. The validity of this explanation for the novel isolates examined in this study, as well as the ability of reference species of the genus *Acidovorax* to synthesize CFA, remains to be determined.

Morphological and physiological traits of the novel isolates were examined as follows. The minimum doubling time attained by the isolated symbionts on ACM broth at 25 °C ranged from 6.5 to 14 h, with values distributed bimodally. Plasmid-free (see above) isolates EF03-1, EF03-2, EF05-1 and EF05-2 showed mean doubling times of 6.5 ± 0.1 h, whereas the remaining isolates showed mean doubling times of 12.1 ± 2.0 h. Doubling times for reference strains growing on ACM broth ranged from 0.7 to 2.5 h. Doubling times and growth rates were calculated as described by Fuchs & Kröger (1999).

Cell morphology was examined by phase-contrast and transmission electron microscopy (TEM). For negative staining, cells were suspended in a 10 mM Mg_2Cl solution. The cell suspension was mixed with an equal volume of 2 % phosphotungstic acid in water and spotted onto coated copper grids. For TEM, cells from late exponential cultures in ACM were fixed in half-strength Karnovsky's fixative ($1 \times$ solution contained 2 % paraformaldehyde and 2.5 % glutaraldehyde) in cacodylate buffer [0.1 M sodium cacodylate; 10 mM $CaCl_2$ (pH 7.3)] overnight at 4 °C. Fixed cells were rinsed in fresh buffer and mixed with an equal volume of 2 % agarose in double-distilled H_2O . Plugs were counterfixed with 1 % OsO_4 in cacodylate buffer for 1 h, dehydrated in an ethanol series and stained with uranyl acetate and lead citrate before sectioning and TEM. For TEM of tissue sections, worms were dissected as above and processed as described by Ballinger *et al.* (1997) with additional post-fixation staining with 2 % uranyl acetate (Ted Pella) prior to embedding. Gram staining and Nile Blue A staining for detection of cellular polyhydroxybuty-

Table 1. Cellular fatty acid methyl ester profiles for *Verminephrobacter eiseniae* sp. nov. and reference strains of the genus *Acidovorax*

Taxa: 1, *Verminephrobacter eiseniae* sp. nov. ($n=8$); 2, *Acidovorax anthurii* ($n=2$; data from Gardan *et al.*, 2003); 3, *A. avenae* subsp. *avenae* ($n=28$; Gardan *et al.*, 2003); 4, *Acidovorax caeni* ($n=3$; Heylen *et al.*, 2008); 5, *Acidovorax defluvi* ($n=2$; Heylen *et al.*, 2008); 6, *Acidovorax delafeldii* ($n=9$; Willems *et al.*, 1990); 7, *Acidovorax facilis* ($n=4$; Willems *et al.*, 1990); 8, *Acidovorax konjaci* ($n=4$; Gardan *et al.*, 2003); 9, *Acidovorax temperans* ($n=12$; Willems *et al.*, 1990); 10, *Acidovorax valerianellae* ($n=10$; Gardan *et al.*, 2003); 11, *Comamonas* spp. ($n=8$; Willems *et al.*, 1990). Values are percent area of named equivalent chain-lengths (ECL). Values are presented as ranges or means ± 1 standard deviation, according to the source of the data.

ECL	1	2	3	4	5	6	7	8	9	10	11
$C_{10:0}$ 3-OH	3.2–4.2	1.1 $\pm$ 0.3	3.1 $\pm$ 0.5	2.1–4.3	3.1–3.9		4.9–6.0	3.0 $\pm$ 0.1	3.1–4.8	2.1 $\pm$ 0.2	4.9–17.3
$C_{12:0}$	2.7–4.5	2.0 $\pm$ 0.2	2.3 $\pm$ 0.1	2.6–3.2	2.2–2.4	2.4–3.0	2.9–3.4	2.5 $\pm$ 0	3.4–5.4	2.9 $\pm$ 0.1	2.6–6.5
$C_{14:0}$	4.8–8.0	1.1 $\pm$ 0.2	2.2 $\pm$ 0.2		1.6	2.9–3.9	2.8–3.6	3.0 $\pm$ 0	0.6–1.9	3.6 $\pm$ 0.1	0.5–3.9
$C_{15:0}$		12.5 $\pm$ 2.6								3.8 $\pm$ 0.9	
$C_{16:1\omega7c}$	17.1–35.5	37.9 $\pm$ 1.6	42.9 $\pm$ 1.0	38.2–41.2	40.6–48.0	35.1–44.1	39.4–45.7	43.1 $\pm$ 0.4	45.1–49.3	46.3 $\pm$ 0.5	22.2–41.5
$C_{16:0}$	26.6–31.0	20.4 $\pm$ 1.7	20.4 $\pm$ 1.7	24.9–26.4	17.7–22.3	21.6–32.8	19.9–25.6	29.3 $\pm$ 0.5	19.6–25.2	29.4 $\pm$ 1.0	14.4–28.3
$C_{17:0}$		5.0 $\pm$ 0.8			4.9–6.3	0.1–0.3	0.1–0.2	0.3 $\pm$ 0	0.1–0.4	0.9 $\pm$ 0.2	0.0–6.8
$C_{17:0}$ cyclo	9.0–25.5		0.3 $\pm$ 0.3			0.1–1.3	0.1–0.9		0–0.1	0.2 $\pm$ 0.3	1.2–15.2
$C_{17:1\omega8c}$		1.0 $\pm$ 0.2								0.1 $\pm$ 0.2	
$C_{17:1\omega6}$		1.4 $\pm$ 0.3			1.4–2.1						
$C_{18:1\omega7c}$	9.5–15.0	11.8 $\pm$ 1.9	15.9 $\pm$ 0.8	22.2–32.0	8.1–11.0	13.9–22.6	18.0–22.9	17.8 $\pm$ 0.6	15.6–22.6	10.3 $\pm$ 0.5	11.6–16.6

rate (PHB) granules were conducted as described by Smibert & Krieg (1994).

When examined by phase-contrast optics, cells from liquid cultures appeared as short, rounded rods, $1.5 \pm 0.2 \times 0.6 \pm 0.2 \mu\text{m}$ in size and single or in doublets (Fig. 2a). Active motility was not observed although cells were occasionally observed pivoting as if tethered to the glass slide by means of flagella. The presence of polar flagella was confirmed by TEM of negatively stained cells (Fig. 2b). PHB granules were observed in all isolated symbionts grown on ACM broth (not shown). Cells of the cultured symbionts were Gram-negative (not shown).

Catalase tests were performed by adding drops of 3% (w/v) hydrogen peroxide to cell suspensions. Oxidase tests were conducted as recommended using a proprietary kit (bioMérieux). All isolated symbionts and other *Acidovorax* reference strains tested positive for oxidase activity. Catalase activity was positive for the *Acidovorax* reference strains but was undetectable for all eight of the novel isolates, irrespective of the age of the cultures.

Fig. 2. Micrographs of cells of the novel strains growing on ACM broth. (a) Phase-contrast; (b) negatively stained electron micrograph, revealing flagellated cells; (c) TEM of fixed cells, illustrating electron-light inclusions characteristic of cultured cells; (d) TEM of earthworm tissue section showing cells of *Verminephrobacter eiseniae* sp. nov. associated with the endothelial microvilli inside the nephridium. Bars, $5 \mu\text{m}$ (a), $0.5 \mu\text{m}$ (b, c, d).

Oxygen relationships were investigated through a 'deep agar' assay (Smibert & Krieg, 1994), modified by substituting agar with 0.4% Phytigel and amending the ACM broth with 1 mg l^{-1} resazurin (redox indicator; $E^0 = -51$). The tubes were incubated at room temperature ($\sim 25^\circ\text{C}$) for 15 days. Tested strains were cultured in duplicate in at least two different experiments. The clear-pink interface (anoxic/oxic boundary) in cultures inoculated with any of the eight isolated earthworm symbionts migrated upwards from $\sim 2.0 \text{ cm}$ deep 10 h post-inoculation to $\sim 1.0 \text{ cm}$ deep, where it stabilized after 6–8 days of incubation. After 15 days incubation, a thin horizon of growth was observed at the said depth; the lower clear zone presented undetectable growth, while the pink region displayed light turbidity (see Supplementary Fig. S2 in IJSEM Online). This pattern was also detected in tubes inoculated with *A. defluvi*. In tubes inoculated with the remaining *Acidovorax* reference species with catalase-positive type strains, the entire tube turned clear 1–2 days post-inoculation and growth was present almost exclusively at the surface of the medium.

The use of alternative electron acceptors was examined using ACM broth amended with 1 mg l^{-1} resazurin and, where appropriate, 10 mM (final concentration) of sodium fumarate, KNO_3 or DMSO. Media were rendered anoxic by storage inside an anaerobic glove box for at least 8 h before dispensing into degassed Balch tubes. Before autoclaving, sealed tubes were gassed with N_2/CO_2 (95/5%). The head space was similarly replaced after autoclaving while the media temperature was maintained at 80°C . Cooled tubes were inoculated (1 : 50) inside the anaerobic glove box with cultures growing on ACM broth and incubated as above. Anoxic conditions were confirmed by the absence of pink colouration. Oxic controls were treated similarly, but exposed to the atmosphere. Growth was defined as final $\text{OD}_{600} > 0.2$. None of the novel symbiotic strains tested were capable of growth on ACM under anoxic conditions, even in the presence of the alternative electron acceptors tested. Recognized species of the genus *Acidovorax*, except for *A. valerianellae* (Gardan *et al.*, 2003) are capable of respiring nitrate.

Determinations of optimal temperature and NaCl concentration for growth were conducted on 2.5 ml of basal defined media with D-mannose (10 mM) and L-glutamine (5 mM) as carbon and nitrogen sources, respectively. These organic substrates have been shown previously to support good growth of the symbionts in defined media (N. Pinel, unpublished data). Cultures for temperature experiments were incubated at 4, 15, 25, 30, 33 and 37°C ; all other incubations were at 25°C . For determination of the optimum pH, L-glutamine was replaced by an equal concentration of urea and MOPS buffer (used for pH values 6.9–7.8) was substituted by MES, HEPES and TRIS buffers for pH ranges of 5.6–6.6, 7.8–8.2, and 8.2–9.0, respectively. Overlapping pH values were included to account for buffer effects on growth rate.

The isolated symbionts grew at temperatures between 10 and 33 °C, with the optimum temperature (highest growth rates) between 25 and 28 °C. NaCl in the medium was not required for growth; the symbionts grew optimally at a sodium concentration between 50 and 60 mM. The latter values agreed with the NaCl equivalent concentration measured from the urine of the earthworm *Lumbricus terrestris* at the ampullar region of the nephridia (Ramsay, 1949). Maximum yield (maximum OD₆₀₀) increased slightly with increasing NaCl up to 90 mM, but dropped significantly at the next tested concentration (120 mM). Growth was observed in media at pH values between 6 and 8.2. The highest growth rates and maximum OD₆₀₀ values were observed at pH values between 7.5 and 8.2, depending on the isolate, but sharp optima were not apparent. Since Tris buffer appeared to inhibit the growth of the novel isolates, an upper pH limit for growth was not determined.

API CH50 galleries (bioMérieux) were used to test the growth of the isolates on a set of 49 carbohydrates over an 80 h incubation period. Media for testing growth on additional carbon and nitrogen sources contained basal defined medium amended with 10 mM of the corresponding carbon or nitrogen source from prepared stocks. The pH was adjusted to between 7.0 and 7.5 if necessary. The medium was filter-sterilized through 0.2 µm membrane filters and aliquoted (200 µl per well) into 96-well microtitre plates. Growth on each substrate was evaluated in triplicate wells on the same plate for each strain. Carbon- or nitrogen-free controls for each strain and uninoculated controls for each substrate were included in every plate. β-Hydroxybutyrate (20 mM) or ammonium chloride (10 mM) served as carbon or nitrogen sources, respectively, for the nitrogen and carbon assimilation tests; ammonium chloride was omitted when testing for growth on amino acids. Cells from mid-exponential ACM cultures were recovered by centrifugation, rinsed once with carbon- and nitrogen-free basal medium and resuspended in fresh carbon- and nitrogen-free medium to an approximate OD₆₀₀ of 1.0. A 10 µl sample of this cell suspension was used to inoculate each well. Plates were sealed with Parafilm to reduce evaporation and incubated at room temperature in the dark on an orbital shaker at 200 r.p.m. Plates of *Acidovorax* reference strains were incubated for 4 days; plates inoculated with the isolated symbionts were incubated for 10 days. Final OD₆₀₀ was measured using a microtitre plate reader. Positive growth was defined as a final OD₆₀₀ > 0.05 after subtraction of the corresponding value from the carbon- or nitrogen-free control. Each substrate was tested using at least two independent replicate plates. Results for the novel symbiotic strains and diagnostic carbon sources are presented in Table 2. A notable difference between the nephridial isolates and free-living species of the genus *Acidovorax* was the greater range of carbohydrates utilized by the symbionts. This may be a characteristic that reflects their symbiotic environment since carbohydrates such as mannose, fucose and galactose, are common in glycosylated surfaces such as those that

may be present in the ampullar epithelium. The use of sugar residues from host surface glycans as a source of energy has been documented in other symbiotic systems (e.g. Sonnenburg *et al.*, 2005).

Starch hydrolysis was assayed on ACMP plates supplemented with 0.2 % (w/v) soluble starch. Starch hydrolysis was not observed for the isolated symbionts, *A. facilis* or *A. temperans*. Other reference strains were not tested. Gelatinase activity was assayed on ACM medium supplemented with 12 % (w/v) gelatin. Gelatinase activity was positive for *A. facilis* and *A. temperans*, but negative for all eight isolated symbionts. Results for other reference strains were obtained from the literature.

Experiments to define growth factor requirements were conducted on basal defined media (see above) without vitamins. For experiments with free-living species of the genus *Acidovorax*, β-hydroxybutyrate replaced D-mannose as the carbon source since some free-living species utilize sugars poorly. Vitamins were supplied as above or added individually at the corresponding concentrations. Amino acids were added at the following final concentrations: glutamate, arginine, aspartate, alanine, valine, threonine (0.5 mM); glutamine, lysine, methionine, asparagine, proline (0.3 mM); and histidine, serine, leucine, tryptophan (0.2 mM). Amino acids were added together, or grouped into six biosynthetic families (Fuchs, 1999) that were then considered as individual treatment units. ACM cultures in mid-exponential growth phase were rinsed with vitamin-free defined medium and transferred into 2.5 ml final volume of fresh vitamin-free defined medium for acclimation and depletion of vitamin traces. After two days, the cultures were inoculated 1:50 into the corresponding treatments.

Biotin alone was required for growth of the novel symbionts in defined media (data not shown). The biotin requirement could be partially alleviated by the addition of a 16-amino acid solution, the addition of aspartate alone, and to a lesser extent by the addition of glutamate (See Supplementary Fig. S3a in IJSEM Online). Of the free-living *Acidovorax* strains tested, *A. defluvii* and *A. temperans* displayed a vitamin dependence for growth in defined mineral medium. Biotin partially fulfilled the requirement of the former, but not that of the latter (see Supplementary Fig. S3b). Biotin participates as a cofactor in carboxylating reactions. One such reaction is the anapleurotic replenishment of oxaloacetate into the tri-carboxylic acid cycle. When supplied with aspartate, an amino acid that can be converted to oxaloacetate, cells may overcome the need for biotin-dependent carboxylation.

Siderophore production was tested for the isolated symbionts and several reference strains. No siderophore production was detected for the earthworm symbionts. A description of the methods and results is available as Supplementary Material and Supplementary Fig. S4 in IJSEM Online.

Table 2. Carbon substrate assimilation and other diagnostic tests for isolates of *Verminephrobacter eiseniae* sp. nov.

Taxa: 1, *Verminephrobacter eiseniae* sp. nov. ($n=8$; data from this study); 2, *A. avenae* subsp. *avenae* ($n=24$; Willems *et al.*, 1992); 3, *A. konjaci* ($n=3$; Willems *et al.*, 1992); 4, *A. defluvii* ($n=8$; Schulze *et al.*, 1999); 5, *A. delafieldii* ($n=14$; Willems *et al.*, 1990); 6, *A. facilis* ($n=4$; Willems *et al.*, 1990); 7, *A. temperans* ($n=18$; Willems *et al.*, 1990); 8, *A. valerianellae* ($n=19$; Gardan *et al.*, 2003). +, Present in 90 % of the strains; –, present in 10 % or less of the strains; D, present in 10–90 % of the strains; ND, not determined. Values in parentheses are the results for the proposed type strain EF01-2^T. The following substrates supported the growth of at least six of the eight symbiotic isolates: L-arabinose, D-fructose, D-fucose, L-fucose, D-glucose, D-mannose, D-galactose, D-xylose, D-lyxose, inositol, D-mannitol, glycerol, lactate, acetate, fumarate, β -hydroxybutyrate, malate, 2-oxoglutarate, pyruvate, succinate, L-alanine, L-asparagine, L-aspartate, L-glutamate, L-glutamine, L-proline. Growth was not supported by the following carbon sources: aconitate, benzoate, citrate, isobutyrate, maleate, L-histidine, L-isoleucine, L-lysine, L-methionine, L-serine, L-threonine, L-valine, glycine, D-adonitol, L-sorbose, dulcitol, D-sorbitol, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-tagatose, L-arabitol, 2-ketogluconate or 5-ketogluconate. Ammonium chloride, urea, L-glutamine, L-glutamate, L-asparagine, L-aspartate, L-alanine, L-arginine, L-ornithine, L-serine and sodium nitrate, but not sodium nitrite, served as nitrogen sources when strains were grown on D-mannose.

Substrate	1	2	3	4	5	6	7	8
Catalase	– (–)	+	+	+	+	+	+	+
Gelatinase	– (–)	D	–	+	D*	+	+	ND
Nitrate reduction	– (–)	+	+	+	+	+	+	–
L-Arabinose	+	+	–	–‡§	+	+	–	ND
D-Fructose	+	+	+	–‡§	+	+	+	+
D-Fucose	+	+	–	–‡	+	–	–	ND
L-Fucose	+	+	–	ND	–	–	–	ND
D-Glucose	+	+	+	–‡§	+	+	+	–
D-Mannose	+	–	+	–‡§	+	+	–	ND
L-Rhamnose	+	–	–	–‡§	–	–	–	ND
D-Ribose	D (+)	D	+	–	+	+	–	–
D-Xylose	+	+	–	–‡§	–	–	–	–
Galactose	+	+	+	ND	–	+	–	ND
DL-Lactate	+	+	+	D	+	–	+	ND
Acetate	D (+)	+	+	+	–	–	+	ND
Aconitate	– (–)	+	+	–	–	–	–	ND
Benzoate	– (–)	–	–	–	–	+	–	ND
Citrate	– (–)	–	–	+	–	–	–	ND
Fumarate	– (–)	+	+	–	+	+	+	+
Glutarate	D (–)	–	–	ND	+	–	+	+
Maleate	– (–)	–	+	–	–	–	–	ND
2-Oxoglutarate	+	+	–	ND	+	–	–	+
Propionate	D (+)	+	+	–	–	+	+	ND
L-Aspartate	+	+	+	–	+	+	+	+
L-Histidine	– (–)	+	–	+	–	+	+	+
L-Isoleucine	– (+)	+	+	+	+	+	+	+
L-Serine	– (–)	+	+	+	+	+	+	ND
L-Threonine	– (–)	–	–	–	+	+	+	ND
L-Valine	– (–)	+	+	+	+	–	+	ND

*Data from Willems *et al.*, 1992.

†Also tested in this study, leading to a delayed reaction (previously reported as negative).

‡Data from this study.

§Schulze *et al.*, 1999.

The values for DNA–DNA hybridization and 16S rRNA gene sequence similarity between the isolated symbionts and the recognized species of the genus *Acidovorax* firmly establish the novel symbionts as representing a novel

species (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). The mean 16S rRNA gene sequence similarity between the earthworm symbionts and either of the other two groups within the genus *Acidovorax* (92.5 ± 0.5 % and

93.3 ± 0.3 % similarity to plant pathogens and other species within the genus, respectively) fell within the range observed at a genus rank (Konstantinidis & Tiedje, 2005b) and was well below that shared among other species in the genus. Gene phylogenies support the position of the earthworm symbionts as exclusive members of a coherent lineage. A sister taxon to this group cannot be consistently ascertained from the available data. In addition to the molecular differences, our proposal for the creation of a new genus considers the distinctive physiological and ecological features of these organisms to be of taxonomic significance, as has been suggested by others (Konstantinidis & Tiedje, 2005a; Stackebrandt *et al.*, 2002).

All of the present data indicate that these bacteria have formed a close alliance with earthworms. The molecular data available from symbionts associated with various earthworm species indicate that these bacteria form a cohesive group related to, but distinct from other species of the genus *Acidovorax*. Their 16S rRNA gene sequence type is rarely detected in surveys of soils or worm culture bedding (see Supplementary Material; S. K. Davidson, M. B. Lund, R. Powell, A. Schramm, D. A. Stahl & S. James, unpublished data). Further, vertical transmission is probably the sole effective transmission mode (Davidson & Stahl, 2006), suggesting that bacteria that are shed into the environment encounter a symbiotic dead end. The symbionts' ability to utilize a larger number of sugars than other described related species, their lack of detectable catalase activity, their inability to respire nitrate, their oxygen relationships and their significantly lower growth rate compared with related species are all characteristics that set them apart from other members of the genus *Acidovorax*. Whole-chromosome differences in genome architecture (unpublished data) further support their evolutionary divergence from the *Acidovorax* group.

Considering the many distinctive ecological, molecular and physiological characteristics described here, we propose the creation of the genus *Verminephrobacter* gen. nov. to include the new, nephridia-associated, betaproteobacterial isolates. Since the available comparative datasets do not resolve the branching order of the symbionts relative to described members of the genus *Acidovorax*, possible paraphyly cannot be excluded. However, deviations from convention to accommodate biological innovation have precedent in systematics (e.g. Adl *et al.*, 2005). The proposed naming reflects the unique ecology of the symbionts and their evolutionary radiation within different earthworm species. We anticipate that this description will provide the basis for future taxonomic revisions of the ecologically and physiologically diverse bacteria now grouped together in the genus *Acidovorax*.

Description of *Verminephrobacter* gen. nov.

Verminephrobacter [Ver.mi.ne.phro'bac.ter. L. n. *vermis* a worm; Gr. n. *nephros* a kidney; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Verminephrobacter* earthworm-kidney (associated) bacteria].

Cells are straight rods, $1.5 \pm 0.2 \times 0.6 \pm 0.2$ µm. They occur singly or in doublets and are flagellated, but motility is seldom observed under standard culturing conditions. Gram-negative. Oxidase-positive. Catalase-negative. Obligate aerobic growth with a preference for low oxygen tensions. Chemo-organotrophic. Oxidative metabolism occurs with oxygen as the terminal electron acceptor. Dissimilatory nitrate reduction is absent. Moderate growth is obtained on complex media containing pyruvate and amino acids. Ammonium salts, urea and nitrate can serve as nitrogen sources in defined media. Biotin is required for growth on defined mineral medium in the absence of amino acids. A cyclopropane-substituted fatty acid (cyclo C_{17:0}) is present as a major component of membrane-derived fatty acid methyl esters in all strains tested. The DNA G+C content is 67 mol%. The genus *Verminephrobacter* comprises nephridium-associated bacterial symbionts of lumbricid earthworm species. The type species is *Verminephrobacter eiseniae*.

Description of *Verminephrobacter eiseniae* sp. nov.

Verminephrobacter eiseniae (ei.se'niae. N.L. gen. n. *eiseniae* of Eisenia, a reference to being the nephridial symbiont of the earthworm species *Eisenia foetida*).

The description is the same as that given for the genus *Verminephrobacter*. Isolated from platings of nephridial homogenates of the earthworm species *Eisenia foetida* onto complex media.

The type strain is EF01-2^T (=ATCC BAA-1489^T=DSM 19286^T).

Note added on revision

The description of *Acidovorax caeni* was published during the late revision stages of this manuscript (Heylen *et al.*, 2008). Data from this paper were incorporated only into Table 1. *A. caeni* groups with *A. delafieldii*, *A. temperans* and *A. defluvii*. The 16S rRNA gene sequences available for *A. caeni* show 93 ± 0.3 % similarity to those of the eight isolates of *V. eiseniae* reported here. The addition of this species to the genus *Acidovorax* does not alter the conclusions of our work.

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