Gallaecimonas pentaromativorans gen. nov., sp. nov., a bacterium carrying 16S rRNA gene heterogeneity and able to degrade high-molecular-mass polycyclic aromatic hydrocarbons

Arturo Rodríguez-Blanco,^{1,2†} Gilles Vétion,^{1,2} Marie-Line Escande,^{1,2} Daniel Delille^{1,2} and Jean-François Ghiglione^{1,2}

Correspondence

Jean-François Ghiglione
ghiglione@obs-banyuls.fr

¹UPMC Univ Paris 06, UMR 7621, Laboratoire ARAGO, Avenue Fontaulé, BP44, F-66650 Banyuls-sur-Mer, France

²CNRS, UMR7621, Laboratoire d'Océanographie Biologique de Banyuls, Avenue Fontaulé, BP44, F-66650 Banyuls-sur-Mer, France

A Gram-negative, rod-shaped, halotolerant bacterium, designated strain CEE_131^T, which degraded high-molecular-mass polycyclic aromatic hydrocarbons of four and five rings, was isolated from intertidal sediment of Corcubion Ria in Cee, A Coruña, Spain. Direct sequencing showed ambiguities and suggested heterogeneity. Cloned 16S rRNA gene sequence PCR products yielded five different sequences varying at five positions. Strain CEE_131^T showed rather distant relationships to its phylogenetically closest neighbours, including the genera *Rheinheimera* and *Serratia*, exhibiting 91 % sequence similarity with *Rheinheimera perlucida* BA131^T and *Serratia proteamaculans* subsp. *quinovora* DSM 4597^T. The major fatty acids were C_{16:1ω7c}, C_{16:0} and C_{18:1ω7c}. The DNA G + C content was 41.7 mol%. On the basis of these distinct phenotypic and genotypic characteristics, strain CEE_131^T is considered to represent a novel species in a new genus in the class *Gammaproteobacteria*, for which the name *Gallaecimonas pentaromativorans* gen. nov., sp. nov. is proposed. The type strain is CEE_131^T (=DSM 21945^T=CECT 7479^T).

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic pollutants that harbour two or more fused benzene rings (Kennish, 1992). Diverse anthropogenic activities, such as the combustion of fossil fuels and oil spills, are responsible for its accumulation in natural environments (Kennish, 1992). Many high-molecular-mass PAHs (i.e. containing from four to seven benzene rings) are mutagenic, carcinogenic or teratogenic for a wide range of organisms including fish, amphibians, birds and mammals (Eisler, 1987).

Micro-organisms that are able to degrade PAHs have been isolated from various environments (Juhász & Naidu, 2000). However, high-molecular-mass PAHs present high

hydrophobicity and have a tendency to adsorb to the organic fraction of soil and sediments (Gauthier *et al.*, 2003). These characteristics are responsible for their low bioavailability to micro-organisms and the resulting persistence in natural environments (Hughes *et al.*, 1997). Two-liquid-phase (TLP) cultures increase bioavailability and biodegradation of hydrophobic substrates (Déziel *et al.*, 1999) and the microbial degradation occurs at the interface and/or in the aqueous phase (Gauthier *et al.*, 2003).

Using a TLP culture containing silicon oil as the water-immiscible phase, we isolated a bacterial strain capable of degrading PAHs, designated strain CEE_131^T, from the Corcubion Ria in north-western Spain, a coastal system that was highly exposed to the Prestige oil spill in 2002.

Strain CEE_131^T harbours 16S rRNA gene sequence heterogeneity. Although not exhaustively studied, 16S rRNA gene heterogeneity at the species or strain level is a common phenomenon (Bosshard *et al.*, 2002). Unexpectedly high levels of intraspecific variation of 16S rRNA gene sequences in GenBank have been found previously (Clayton *et al.*, 1995). Other studies have dealt with heterogeneity in 16S rRNA gene sequences (Bricker, 2000; Cilia *et al.*, 1996; Liefting *et al.*, 1996; Martinez-Murcia

[†]Present address: Department of Microbiology, Institute of Aquaculture, University of Santiago de Compostela, Santiago de Compostela, Spain.

Abbreviations: CE-SSCP, capillary electrophoresis single-strand conformation polymorphism; PAH, polycyclic aromatic hydrocarbon; TLP, two-liquid-phase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain CEE_131^T are FM955223–FM955227.

A micrograph of sectioned cells of strain CEE_131^T obtained by using transmission electron microscopy and graphs showing the effects of temperature, pH and salinity on the growth of the strain are available as supplementary material with the online version of this paper.

et al., 1999; Ninet *et al.*, 1996; Nübel *et al.*, 1996; Pettersson *et al.*, 1998; Reischl *et al.*, 1998).

For the isolation of strain CEE_131^T, collected sediment (5 g) was used to inoculate 50 ml MM2 broth [containing per litre of aged seawater (ZoBell, 1946): 18 mM (NH₄)₂SO₄, 1 µM FeSO₄·7H₂O and 100 µl 1 M KH₂PO₄/Na₂HPO₄ buffer solution], which was supplemented with 1000 p.p.m. of a mixture of equal quantities of naphthalene, fluorene, phenanthrene, fluoranthene, pyrene and benzo[a]pyrene. Silicon oil was added at 20 % (v/v). The culture was incubated at 25 °C with orbital shaking for 9 months, during which time 5 ml culture was transferred to 50 ml fresh medium every 3 months. Bacterial strains were isolated at 25 °C for 48 h on marine broth solid medium (marine agar 2216; MA, Difco) from which their PAH-degrading capacity was elucidated following the spray-plating method of Kiyohara *et al.* (1982). Strain CEE_131^T formed a degradation halo on solid medium sprayed with pyrene after 30 days of incubation at 25 °C.

The capacity of strain CEE_131^T to degrade different PAHs was tested in liquid batch experiments over a period of 15 days. Cells were precultured in marine broth 2216 (MB; Difco) at 25 °C for 12 h. The cells were then centrifuged at 5812 g for 15 min before being resuspended as triplicate cultures in MM2 broth containing (ml⁻¹): 50 µg pyrene and benzo[a]pyrene. Uninoculated controls were kept to estimate abiotic loss of PAHs. After 15 days of incubation at 25 °C, PAHs were extracted with dichloromethane and analysed by using GC performed on a Perichrom ST200 chromatograph equipped with a flame-ionization detector using a Zebron capillary GC column (ZB-5ms; Phenomenex). The conditions for GC were as follows: injector temperature, 300 °C; detector temperature, 320 °C; column temperature, 70 °C for 2 min; then raised using a linear temperature gradient of 10 °C min⁻¹ to a final temperature of 300 °C, which was maintained for 15 min; 1 µl sample was injected with a split ratio of 100:1; and the flow rate of the carrier gas (helium) was 1 ml min⁻¹. PAHs were identified by comparing the relative retention times with pure standards (Accustandard). Loss of PAHs due to the extraction procedure was estimated using docosane (Fluka) as the internal standard. During 15 days, strain CEE_131^T degraded 12.14 µg pyrene ml⁻¹ and 10.93 µg benzo[a]pyrene ml⁻¹ (24.29 and 21.87 % of the initial quantity, respectively).

The cellular morphology of the isolate was observed by using transmission electron microscopy. For negative staining, strain CEE_131^T was fixed with 2.5 % glutaraldehyde and stained with 0.1 % uranyl acetate. For the observation of sectioned cells, glutaraldehyde fixation was followed by post-fixation with 1 % OsO₄ before the samples were dehydrated gradually, embedded in Epon and visualized at 80 kV with a Hitachi 7500 microscope. The potassium hydroxide test (Powers, 1995) was used to determine whether strain CEE_131^T was Gram-negative; most cell walls were lysed when observed, which indicated

Fig. 1. TEM micrograph of a negatively stained cell of strain CEE_131^T grown in MB for 12 h at 25 °C, showing the presence of one polar flagellum. Bar, 500 nm.

that the strain was Gram-negative. The temperature and pH ranges for growth, at 20, 25, 30 and 37 °C and pH 3–10, respectively, were tested in MB. The salinity requirements of the strain were tested in modified MB (Sohn *et al.*, 2004) supplemented with 0–10 % (w/v) NaCl. Cells of strain CEE_131^T were Gram-negative, rod-shaped and presented one polar flagellum (Fig. 1). A transmission electron microscope image is shown in Supplementary Fig. S1 (available in IJSEM Online). Cells were 1.4–2.9 µm long and 0.3–0.5 µm wide. Colonies were circular, convex and grey-coloured on MA. Growth was observed at 20–37 °C (optimum, 30 °C), at pH 5–10 (optimum, pH 5–8) and with 0–7 % NaCl (optimum, 1–2 % NaCl) (Supplementary Fig. S2, in IJSEM Online).

Physiological and biochemical characterization was performed using API 20NE (bioMérieux) and Biolog GN2 plates, according to the manufacturers' instructions. Oxidase activity was found to be negative. API 20NE positive reactions were observed for hydrolysis of aesculin and gelatin and assimilation of D-glucose, L-arabinose, N-acetylglucosamine, maltose, malate and citrate. Biolog GN2 plates showed positive results for utilization of the following carbon sources: α-cyclodextrin, dextrin, glycogen, Tweens 40 and 80, N-acetyl-D-glucosamine, L-arabinose, cellobiose, α-D-glucose, maltose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid, α-, β- and γ-hydroxybutyric acids, itaconic acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-proline, L-serine, L-threonine, urocanic acid, thymidine and α-D-glucose 1-phosphate.

Fatty acid methyl esters were obtained by acid transmethylation with a H₂SO₄/methanol/chloroform mixture from cells cultured in MB according to Indarti *et al.* (2005) using nonadecanoic acid (Larodan Fine Chemicals) as the internal standard. Methyl esters in hexane (1 µl) were analysed by GC-MS (Saturn 2100T; Varian) by using a fused silica capillary column (VF-Wax ms; Varian). The conditions for GC-MS were as follows: injector temperature 260 °C; MS detector temperatures 230 °C (for transfer line and the ion trap) and 50 °C (for the manifold); oven temperature 150 °C for 30 min, raised to 180 °C at 2 °C min⁻¹, raised to 212 °C at 1 °C min⁻¹, maintained for 5 min, then raised to 250 °C at 5 °C min⁻¹, which was maintained for 15 min. The flow rate of the carrier gas (helium) was 1 ml min⁻¹.

Fatty acid identification was made by comparison of retention times and MS spectra with different standard solutions: Supelco 37, PUFA 1, PUFA 3, BAMEs (Supelco) and Qalmix Fish S (Larodan Fine Chemicals). The major fatty acids of strain CEE_131^T were C_{16:1}ω7c (30.43 %), C_{16:0} (29.88 %) and C_{18:1}ω7c (17.14 %). The following fatty acids were detected as minor components: C_{14:0} (3.54 %), C_{12:0} 3-OH (3.53 %), C_{18:0} (3.33 %), C_{12:0} (3.31 %), iso-C_{16:0} (1.84 %), C_{17:0} (1.24 %), C_{17:1}ω7c (1.12 %), C_{10:0} (0.81 %), anteiso-C_{15:0} (0.7 %), C_{15:0} (0.6 %), iso-C_{17:0} (0.55 %), C_{18:2}ω6c (0.52 %) and C_{18:1}ω9c (0.50 %). The fatty acids with different proportions in strain CEE_131^T to those in the closest phylogenetically related genus, *Rheinheimera*, are shown in Table 1.

For 16S rRNA gene sequencing, total genomic DNA was extracted using a DNeasy Tissue kit (Qiagen), according to

Table 1. Differential characteristics of strain CEE_131^T and members of the genus *Rheinheimera*

Rheinheimera species: *R. baltica* (grown on half-strength MA; Brettar *et al.*, 2002); *R. pacifica* (MA; Romanenko *et al.*, 2003); *R. perlucida* (MA; Brettar *et al.*, 2006); *R. chironomi* (TSA; Halpern *et al.*, 2007); *R. aquimaris* (MA; Yoon *et al.*, 2007); *R. texasensis* (R2A agar; Merchant *et al.*, 2007); *R. soli* (MA; Ryu *et al.*, 2008); *R. tangshanensis* (TSA; Zhang *et al.*, 2008). –, Not detected.

Characteristic	Strain CEE_131 ^T	<i>Rheinheimera</i> species
Fatty acids (%)		
C _{14:0}	3.54	0–2.28
C _{16:0}	29.88	7.80–25.1
C _{18:0}	3.33	0–2.0
anteiso-C _{15:0}	0.70	0–0.28
iso-C _{17:0}	0.55	0–0.19
C _{17:1} ω7c	1.12	–
C _{17:1} ω8c	–	3.8–15.52
C _{18:1} ω7c	17.14	4.96–15.87
C _{18:2} ω6c	0.52	–
DNA G + C content (mol%)	41.7	47.0–50.5

the manufacturer's instructions. 16S rRNA gene sequence amplification was performed with the primers SAdir forward [5'-AGAGTTTGATCATGGCTCAG-3'; *Escherichia coli* 16S rRNA gene sequence positions 8–27; numbering according to Brosius *et al.* (1981)] and S17 reverse (5'-GTTACCTTGTTACGACTT-3'; positions 1491–1508). The polymerase chain reaction was performed as described by Rodríguez-Blanco *et al.* (2009). The amplification products were cloned using a TOPO TA cloning kit for sequencing (Invitrogen), according to the manufacturer's instructions. Plasmid DNA was extracted from 70 colonies using the Wizard SV 96 Plasmid DNA Purification System (Promega). In order to find possible differences in 16S rRNA gene sequences, we used capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) to compare the highly variable V3 region (positions 329–533) of the 70 clones. CE-SSCP was performed as described by Rodríguez-Blanco *et al.* (2009). Five patterns were identified on the basis of different CE-SSCP migration behaviours, one of them being dominant (94 %). 16S rRNA gene sequences of representative clones for these five patterns were then determined (Genoscreen, France). 16S rRNA gene sequences in which variations were found are shown in Table 2; nearly half of them were found in the V3 region.

Phylogenetic identification was performed using BLAST (<http://www.ncbi.nlm.nih.gov/blast>). Phylogenetic trees were obtained using PHYLO WIN according to the neighbour-joining and maximum-parsimony methods (Galtier *et al.*, 1996). Bootstrap analysis was performed based on 500 replications with the Kimura two-parameter correction for the distances. Phylogenetic affiliations showed clear affiliation with members of the *Gammaproteobacteria*. The closest related genus was *Rheinheimera* (Fig. 2), from which *Rheinheimera perlucida* LMG 23581^T was the closest species, sharing 91 % similarity with strain CEE_131^T.

Table 2. 16S rRNA gene sequence variations in strain CEE_131^T

Positions are given according to the 16S rRNA gene sequence of *E. coli* (Brosius *et al.*, 1981).

Clone no.	Position										
	85	349*	397–398*	428*	464*	509*	812	1010	1017	1209	1317
32	T	G	TG	C	A	A	T	C	G	T	A
33	T	G	CA	T	A	A	T	C	G	C	G
36	C	A	TG	T	A	A	T	T	A	C	A
42†	T	G	TG	T	G	A	C	C	G	C	A
45	T	G	TG	T	A	G	T	C	G	C	A

*Positions inside the V3 region.

†Most frequent 16S rRNA gene sequence in strain CEE_131^T genome.

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain CEE_131^T and related strains. Bootstrap percentages at branch nodes are shown only for those also retrieved in the maximum-parsimony tree. Bar, 0.1 substitutions per nucleotide position.

The DNA G+C content was determined by HPLC as follows. Cells were disrupted using a French press and DNA was purified on hydroxyapatite (Cashion *et al.*, 1977). After hydrolysis with P1 nuclease and dephosphorylation with bovine alkaline phosphatase, the resulting deoxyribonucleosides were analysed by using HPLC (Shimadzu, Japan). A sample of 10 µl was treated at 45 °C with 0.3 M (NH₄)H₂PO₄/acetonitrile, 40:1 (v/v), pH 4.4 (adapted from Tamaoka & Komagata, 1984). Calibration was done using non-methylated phage lambda DNA (Sigma) and the G+C content was calculated from the ratio of deoxyguanosine and deoxythymidine as described by Mesbah *et al.* (1989). The DNA G+C content of strain CEE_131^T was 41.7 mol%.

On the basis of strain CEE_131^T having different phenotypic and genotypic properties to those of previously described taxa, such as the fatty acid composition, the phylogenetic distances based on the 16S rRNA gene sequence similarities with its closest relatives and the DNA G+C content, we propose a new genus in the class *Gammaproteobacteria*: *Gallaecimonas* gen. nov. Strain CEE_131^T is proposed to represent the type strain of the novel species *Gallaecimonas pentaromativorans* gen. nov., sp. nov.

Description of *Gallaecimonas* gen. nov.

Gallaecimonas (Gal.la.e.ci.mo'nas. L. n. *Gallaecia* of Galicia, a region of north-west Spain; L. fem. n. *monas* a

monad, unit; N.L. fem. n. *Gallaecimonas* single microbe isolated from Galicia).

Cells are Gram-negative, flagellated rods. Oxidase-negative. Major fatty acids are C_{16:0}, C_{16:1ω7c} and C_{18:1ω7c}. Minor fatty acids are C_{14:0}, C_{12:0} 3-OH, C_{18:0}, C_{12:0} iso-C_{16:0}, C_{17:0}, C_{17:1ω7c}, C_{10:0}, anteiso-C_{15:0}, C_{15:0}, iso-C_{17:0}, C_{18:2ω6c} and C_{18:1ω9c}. The DNA G+C content of the type strain of the type species is 41.7 mol%. The type species is *Gallaecimonas pentaromativorans*.

Description of *Gallaecimonas pentaromativorans* sp. nov.

Gallaecimonas pentaromativorans (pen'ta.ro.ma'ti.vo.rans. Gr. numeral *penta* five; L. n. *aroma* -atis spice; L. v. *voro* devour; N.L. part. adj. *pentaromativorans* degrading/devouring aromatic compounds with five rings).

The following properties are also observed in addition to those given in the genus description. Colonies are circular, convex and grey-coloured on MA. Growth is observed at 20–37 °C (optimum, 30 °C) and at pH 5–10 (optimum, pH 5–8). Does not require NaCl for growth and can tolerate up to 7% NaCl (optimum, 1–2% NaCl). Hydrolyses aesculin and gelatin and assimilates D-glucose, L-arabinose, N-acetylglucosamine, maltose, malate and citrate. Oxidizes α-cyclodextrin, dextrin, glycogen, Tweens 40 and 80, N-acetyl-D-glucosamine, L-arabinose, cellobiose,

α -D-glucose, maltose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid, α -, β - and γ -hydroxybutyric acids, itaconic acid, α -ketoglutaric acid, DL-lactic acid, malonic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-proline, L-serine, L-threonine, urocanic acid, thymidine and α -D-glucose 1-phosphate. Degrades pyrene and benzo[a]pyrene.

The type strain, CEE_131^T (=DSM 21945^T=CECT 7479^T), was isolated from intertidal sediment of Corcubion Ria in Cee, A Coruña, Spain.

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