***Opitutus terrae* gen. nov., sp. nov., to accommodate novel strains of the division 'Verrucomicrobia' isolated from rice paddy soil**

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Three strains of obligately anaerobic bacteria were isolated from rice paddy soil microcosms. Comparative analysis of the 16S rRNA genes showed that these novel isolates have identical gene sequences and are members of the division 'Verrucomicrobia'. The novel strains are phenotypically and phylogenetically distinct from species described previously. One strain, PB90-1^T, was characterized in more detail. The cells are cocci and are motile by means of a flagellum. Catalase and oxidase activities are absent. Growth-supporting substrates include mono-, di- and polysaccharides, while alcohols, amino acids and organic acids do not support growth. Propionate and acetate are the major end-products of fermentation. Nitrate is reduced to nitrite, but other external electron acceptors are not utilized. The G+C content of the genomic DNA is 74 mol%. This strain represents a taxon that has not yet been formally recognized, for which the name *Opitutus terrae* gen. nov., sp. nov. is proposed. The type strain is PB90-1^T (= DSM 11246^T).

Keywords: *Opitutus terrae*, 'Verrucomicrobia', 16S rRNA gene, anaerobe

INTRODUCTION

Molecular ecological studies, based on the recovery of 16S rRNA gene sequences from many different habitats, have revealed a great diversity of bacterial groups encompassing many as-yet uncultivated organisms, often only distantly related to currently known isolates. One of the phyla or divisions of bacterial descent with very few cultivated representatives, but for which 16S rRNA gene sequences reveal a much wider phylogenetic diversity than that known from cultivation studies, is the division 'Verrucomicrobia' (Hedlund *et al.*, 1997; Hugenholtz *et al.*, 1998). We have recently reported the detection of members of this lineage in a library of cloned 16S rRNA genes from anoxic rice paddy soil (Hengstmann *et al.*, 1999) and reported the isolation of very close relatives of these molecularly detected organisms in parallel cultivation studies from the same soil sample (Chin *et al.*, 1999). Our previous investigations

(Hengstmann *et al.*, 1999; Chin *et al.*, 1999) suggested that the novel isolates, strains PB90-1^T, PB90-3 and ACB90, are phylogenetically distinct from currently described species of bacteria. We have characterized one of these strains further and describe it as the type strain of a new species and genus, *Opitutus terrae* gen. nov., sp. nov.

METHODS

Cultivation and characterization. Strain PB90-1^T (= DSM 11246^T) was isolated from rice paddy soil microcosms in an earlier study (Chin *et al.*, 1999) and was grown in sulfide-reduced, bicarbonate-buffered mineral medium SM supplemented with vitamins as described by Chin *et al.* (1999). Growth substrates and other supplements were prepared as neutralized (with NaOH or HCl, as required), 0.2–2 M or 5% (w/v) stock solutions and were sterilized by autoclaving or, in the case of heat-labile compounds and sugars, by filtration (pore size 0.2 µm). Substrates were added to sterile media just before inoculation. L-Isomers of organic and amino acids and D-isomers of sugars were used. Amorphous cellulose and pectin were prepared as described previously (Janssen *et al.*, 1997). Unless noted otherwise, cultures were grown at pH 7.2 and at 30 °C, in the dark, with 4 mM glucose as the growth substrate.

The methods used for checking culture purity and for phenotypic characterization have been described previously

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The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain PB90-1^T is AJ229235.

(Janssen *et al.*, 1997; Chin *et al.*, 1998, 1999). The G+C content of the genomic DNA was determined by HPLC (Janssen *et al.*, 1996).

Electron microscopy Formvar-coated copper grids were floated on a drop of bacterial culture to allow cells to adhere. The grids were then floated on a drop of 10 mM potassium phosphate buffer (pH 7.4) containing 4% (w/v) paraformaldehyde (dissolved just prior to use) to fix the cells and then 'washed' by floating on a drop of sterile water. The cells were then stained by floating the grid on a drop of 1% (w/v) uranyl acetate (pH 4.0) and then washed again on water. The cells were examined with a CM120 BioTWIN transmission electron microscope (Philips).

Comparative 16S rRNA gene sequence analysis. The phylogenetic position of strain PB90-1^T in relation to representative members of the division '*Verrucomicrobia*' was deduced from evolutionary distances (Jukes & Cantor, 1969) and a neighbour-joining algorithm (Saitou & Nei, 1987), as described previously (Hengstmann *et al.*, 1999), implemented in the ARB program package (developed by O. Strunk and W. Ludwig, Technische Universität München; <http://www.mikro.biologie.tu-muenchen.de/pub/ARB/>). All reference sequences used for the treeing analysis had at least 1400 unambiguously determined nucleotide sequence positions. The only exceptions were the cloned 16S rRNA gene sequences RB14 and RB24 from uncultured bacteria, for which 928 and 984 nucleotide sequence positions, respectively, were available. The dendrogram was constructed on the basis of a consideration of 1281 alignment positions that contained identical nucleotides in at least 50% of the 16S rRNA gene sequences compared. The evolutionary distances between pairs of sequences were calculated by taking into account only those alignment positions at which both sequences had an unambiguously determined nucleotide.

RESULTS AND DISCUSSION

Morphological and cytological characteristics

Cells of strain PB90-1^T were cocci, 0.4–0.6 µm in diameter and motile by means of a flagellum (Fig. 1). Diplococci were commonly observed, but only one cell of these ever had a flagellum. Sometimes, short chains of three or four cells were observed, which had the

Fig. 1. Electron micrograph of a negatively stained preparation of strain PB90-1^T, showing a dividing cell with a single flagellum. Bar, 200 nm.

appearance of short rods when viewed with the phase-contrast microscope.

Cells of strain PB90-1^T stained Gram-negative. The aminopeptidase reaction and KOH test were both positive. No spores were observed in fresh cultures, in cultures grown with 50 mM glucose or in cultures grown with 4 mM glucose with increased concentrations of CaCl₂·2H₂O (0.3 g l⁻¹), MnCl₂·4H₂O (50 mg l⁻¹) and thiamine chloride hydrochloride (30 mg l⁻¹) and supplemented with 10% (v/v) soil extract. The colonies in agar deeps were unpigmented and granular in appearance.

The G+C content of the genomic DNA of strain PB90-1^T was 73.7 ± 0.3 mol% (mean ± SD; *n* = 3).

Nutritional characteristics

Mono-, di- and polysaccharides supported growth of strain PB90-1^T. Growth was observed with glucose, fructose, galactose, mannose, galacturonic acid, man- nitol, arabinose (all tested at 4 mM), cellobiose, maltose, sucrose, lactose, melibiose (all tested at 2 mM), xylan, pectin and starch (all tested at 0.1%, w/v). Those substrates tested and found not to support growth were xylose, ribose, sorbose, methyl α-glucopyranoside (all tested at 2 mM), cellulose, chitin, arabinogalactan (all tested at 0.1%, w/v), pyruvate, lactate, fumarate, malate, tartrate, citrate, crotonate, glycerol (with or without 2 mM acetate), aspartate, alanine, serine, leucine, isoleucine, glutamate, proline, lysine (all tested at 20 mM) and H₂ (60 kPa with or without 2 mM acetate).

Propionate and acetate were the major end-products of fermentation. Smaller amounts (≤ 0.2 mol mol⁻¹ monosaccharide or other growth substrate used) of succinate, lactate, ethanol and H₂ were also formed. On pectin, methanol was also produced. Strain PB90-1^T used nitrate as an electron acceptor with glucose and the sole product of nitrate reduction was nitrite. Sulfur, sulfate, sulfite, thiosulfate and fumarate were not used as electron acceptors.

Growth and biochemical properties

Strain PB90-1^T behaved as an obligate anaerobe in oxygen-tolerance tests. No growth occurred on aerobically incubated agar plates with nutrient broth and glucose. Strain PB90-1^T was unable to grow in the upper 20 mm of agar deep cultures under a headspace of N₂/CO₂ to which 20 kPa O₂ was added.

Strain PB90-1^T was able to grow at pH values from 5.5 to 9.0, with maximum growth rates being achieved at pH 7.5–8.0. Growth was possible at temperatures of 10 to 37 °C, but no growth occurred at 4 or 40 °C. Growth was possible in growth media supplemented with 30 g NaCl l⁻¹ but not with 40 g NaCl l⁻¹.

Fig. 2. Neighbour-joining dendrogram showing the phylogenetic position of strain PB90-1^T (*Opitutus terrae*) in relation to representative 16S rRNA gene sequences that encompass the currently known phylogenetic diversity of the division 'Verrucomicrobia'. Validly described species and cultivated strains are shown in bold. The habitat source is indicated before the name of cloned sequences of as-yet uncultivated organisms. EMBL/GenBank/DDBJ accession numbers are listed in parentheses. The numbers before the interior branch points indicate those five major lineages within the division 'Verrucomicrobia' for which the taxonomic level of subdivision has been proposed (Hugenholtz *et al.*, 1998). These five major lineages were recovered in all treeing analyses with respective bootstrap values above 65 and 90% in neighbour-joining (first number under branch point) and parsimony (second number under branch point) analyses (1000 data resamplings). Scale bar, 0.10 changes per nucleotide position.

Strain PB90-1^T hydrolysed aesculin, but did not hydrolyse gelatin or urea. No catalase or oxidase activities were detected.

Phylogenetic relationships

Strains PB90-1^T, PB90-3 and ACB90 have identical full-length 16S rRNA gene sequences (Hengstmann *et al.*, 1999). Their membership of the division 'Verrucomicrobia' (Hedlund *et al.*, 1997) has been reported previously (Hengstmann *et al.*, 1999). The division 'Verrucomicrobia' is represented by only a few cultivated species and the majority of members of this division are known only as 16S rRNA gene sequences recovered from a variety of habitats (Hedlund *et al.*, 1997; Hugenholtz *et al.*, 1998; Zwart *et al.*, 1998; O'Farrell & Janssen, 1999). The closest cultivated relatives of the novel strains are strains VeCb1, VeGlc2 and VeSm13 (Janssen *et al.*, 1997; Hengstmann *et al.*, 1999). The latter three strains share 16S rRNA gene sequence similarities with PB90-1^T, PB90-3 and ACB90 of between 96 (VeCb1, VeGlc2) and 97% (VeSm13) and thus all of these strains could be members of the same genus. The similarity values between the 16S rRNA gene sequences of strains PB90-1^T, PB90-3 and ACB90 and those of members of the genera *Verrucomicrobium* (Schlesner, 1987) and *Prosthecobacter* (Staley *et al.*, 1976; Hedlund *et al.*, 1997), the only two named genera of the division 'Verrucomicrobia' with cultivated strains, range from 79.8 to 80.1%. These rather large phylogenetic distances are the basis for the inclusion of the novel strains in the recently proposed subdivision 4 (Hugenholtz *et al.*, 1998) of the division 'Verrucomicrobia', while members of the genera *Verrucomicrobium* and *Prosthecobacter* form a coherent cluster within subdivision 1 (Hugenholtz *et al.*, 1998) (Fig. 2). *Verrucomicrobium spinosum* and

Prosthecobacter species are aerobic bacteria with rod-shaped cells and their phenotypic characteristics (Staley *et al.*, 1976; Schlesner, 1987; Hedlund *et al.*, 1997) are quite different from those of the novel isolates. The recently described ectosymbiont of the hypotrich ciliate *Euplotidium arenarium* (Petroni *et al.*, 2000) appears to be a member of subdivision 4 (Fig. 2), but it has not yet been named and is not available in pure culture. This ectosymbiont shares 16S rRNA gene sequence similarity of 83% with strains PB90-1^T, PB90-3 and ACB90, while the similarity values with 16S rRNA gene sequences representative of the other subdivisions (Fig. 2) range between 74 and 78%. The uncultured endosymbionts of nematodes reported recently by Vandekerckhove *et al.* (2000), assigned to the genus *Xiphinematobacter*, are members of subdivision 2 and hence are phylogenetically well separated from the three novel strains (not shown in Fig. 2).

Strains PB90-1^T, PB90-3 and ACB90 are phenotypically very similar (Chin *et al.*, 1999). All three strains have no close phylogenetic relatives that have been formally named in the taxonomic literature and are phenotypically distinct from previously described genera and species of the division 'Verrucomicrobia'. Accordingly, we designate strain PB90-1^T as the type strain of a new species in a new genus, which we name *Opitutus terrae* gen. nov., sp. nov. Strains PB90-3 and ACB90 appear to belong to the same species.

Description of *Opitutus* gen. nov.

Opitutus (O.pi.tu'tus. L. fem. n. *Ops* a Roman Earth and harvest goddess; L. part. adj. *tutus* protected; N.L. masc. n. *Opitutus* the one protected by *Ops*).

Cells are cocci, motile by means of flagella. No spores are formed. Growth-supporting substrates include

mono-, di- and polysaccharides. Alcohols, amino acids and organic acids do not support growth. Propionate and acetate are the major end-products of fermentation. Nitrate can be reduced to nitrite. Obligately anaerobic metabolism. No catalase or oxidase activity. On the basis of comparative analysis of the 16S rRNA genes, members of this genus belong phylogenetically to subdivision 4 (*sensu* Hugenholtz *et al.*, 1998) of the division 'Verrucomicrobia'. The type species is *Opitutus terrae*.

Description of *Opitutus terrae* sp. nov.

Opitutus terrae (ter'rae. L. gen. n. *terrae* of the earth). Cells are cocci, 0.4–0.6 µm in diameter, motile by means of a flagellum. No spores are formed. The colonies in agar deeps are unpigmented and granular in appearance. The G+C content of the genomic DNA of strain PB90-1^T is 74 mol %. The type strain grows with glucose, fructose, galactose, mannose, galacturonic acid, mannitol, arabinose, cellobiose, maltose, sucrose, lactose, melibiose, xylan, pectin and starch. Xylose, ribose, sorbose, methyl α-glucopyranoside, cellulose, chitin, arabinogalactan, pyruvate, lactate, fumarate, malate, tartrate, citrate, crotonate, glycerol (with or without acetate), aspartate, alanine, serine, leucine, isoleucine, glutamate, proline, lysine and H₂ (with or without acetate) do not support growth of the type strain. Propionate and acetate are the major end-products of fermentation. Succinate, lactate, formate, ethanol and H₂ are also produced. Methanol is also formed from pectin. Nitrate is reduced to nitrite. Sulfur, sulfate, sulfite, thiosulfate and fumarate are not used as electron acceptors. Obligately anaerobic. The type strain grows at pH values of 5.5 to 9.0, with maximum growth rates at pH 7.5–8.0. Growth of the type strain is possible at temperatures of 10 to 37 °C, but not at 4 or 40 °C. Aesculin is hydrolysed, but gelatin and urea are not. The type strain is PB90-1^T (= DSM 11246^T).

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