Fermentative bacteria from estuarine mud: phylogenetic position of *Acidaminobacter hydrogenoformans* and description of a new type of Gram-negative, propionigenic bacterium as *Propionibacter pelophilus* gen. nov., sp. nov.

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The phylogenetic positions of two strains of fermentative bacteria that had been isolated from the highest positive tubes inoculated with serial dilutions of estuarine mud in agar media with either glutamate or aspartate as substrate were determined by comparative sequence analysis of their 16S rRNA genes. The strain isolated with glutamate (glu 65) utilized several substrates, including a number of amino acids but no sugars. The degradation of certain substrates was enhanced by or dependent upon co-cultivation with a hydrogen-utilizing partner. In earlier work this strain was assigned to the new genus and species *Acidaminobacter hydrogenoformans*. On the basis of its 16S rRNA gene sequence *Acidaminobacter hydrogenoformans* has now been identified as a member of cluster XI of the *Clostridium* subphylum with *Clostridium halophilum* as its closest relative. The aspartate-fermenting strain asp 66^T was a Gram-negative, rather aerotolerant anaerobe which utilized a wide range of substrates in a propionic fermentation and had the ability to fix molecular nitrogen. Strain asp 66^T was shown to be a new member of the β -subclass of the *Proteobacteria* with *Azoarcus* sp. strain 6a3 and *Rhodocyclus tenuis* as its closest relatives. It is described as *Propionibacter pelophilus* gen. nov., sp. nov., with the type strain asp 66^T (= DSM 12018^T).

Keywords: *Propionibacter* gen. nov., *Acidaminobacter*, propionic fermentation

INTRODUCTION

In 1982 in the laboratory of one of us two new types of fermentative bacteria were isolated from mud of the Ems-Dollard estuary at the border between Germany and The Netherlands. These rod-shaped bacteria were obtained from the highest positive dilutions in anoxic agar media with glutamate or aspartate as the substrate. The glutamate-fermenting strain (glu 65) stained Gram-negative; electron micrographs, however, showed that it did not have a typically Gram-negative cell envelope. It fermented several substrates but some of them were only utilized in co-cultures with

a hydrogenotrophic partner. Furthermore, in co-cultures the fermentation of glutamate was faster and it was shifted towards the conversion of glutamate to propionate, CO₂ and hydrogen. This strain was described as the type strain of *Acidaminobacter hydrogenoformans* gen. nov., sp. nov. (Stams & Hansen, 1984). The aspartate-fermenting strain asp 66^T was Gram-negative; in addition to aspartate, it fermented several substrates (sugars, sugar alcohols, dicarboxylic acids) in a propionic fermentation. It grew in mineral media with a single substrate, could fix N₂, and was rather oxygen-tolerant but did not grow in well-aerated cultures (Hansen *et al.*, 1990). It was speculated that despite its use of a much larger diversity of substrates strain asp 66^T might be related to *Pelobacter propionicus*, which is now known to belong to the δ -subclass of the *Proteobacteria* (Evers *et al.*, 1993) but

The GenBank accession numbers for the 16S rRNA gene sequences of *Propionibacter pelophilus* and *Acidaminobacter hydrogenoformans* are AF016690 and AF016691, respectively.

whose phylogenetic position was unknown when strain asp 66^T was characterized.

In this paper we describe the phylogenetic position of strain asp 66^T, show that it belongs to the β -subclass of the *Proteobacteria* and describe it as the type strain of a new genus and species for which we propose the name *Propionibacter pelophilus* gen. nov., sp. nov. We also show that on the basis of the 16S rRNA gene sequence *Acidaminobacter hydrogenoformans* groups with Gram-positive anaerobes belonging to cluster XI of the *Clostridium* subphylum.

METHODS

Cultivation of the strains. Strain asp 66^T (DSMZ 12018^T) was grown in minimal bicarbonate-buffered media with aspartate or glucose as carbon and energy source as described previously (Hansen *et al.*, 1990). *Acidaminobacter hydrogenoformans* (DSMZ 2784^T) was grown in a medium with 20 mM glutamate as described (Stams & Hansen, 1984). Culture purity and identity were verified by phase-contrast microscopy and analysis of the fermentation products.

Comparative sequence analysis of 16S rRNA genes. Chromosomal DNA was isolated as described previously (Van der Maarel *et al.*, 1996). The 16S rRNA genes were amplified by PCR using *Pwo* DNA polymerase as recommended by the manufacturer (Boehringer) using the oligonucleotides 5' ACCTAATACGACTACTATAGGGAGAGTTTGATC-CTGGCTCAG 3' and 5' ATTGTAACACGACGGCC-AGTGGTTACCTTGTACGACTT 3'. The 3' ends of

these primers are complementary to conserved rRNA sequences corresponding to positions 8–27 and 1492–1510, respectively, of the *Escherichia coli* rRNA gene; the 5' ends of these primers are complementary to two universal fluorescently labelled sequencing primers. The reaction mixture was pre-incubated at 95 °C for 5 min and subsequently subjected to 27 cycles of 94 °C for 45 s, 52 °C for 45 s, 72 °C for 120 s followed by an incubation at 72 °C for 7 min. The PCR product was purified using Qiaquick spin columns (Qiagen). The nucleotide sequence of the amplified 16S rRNA genes was determined using an ALF Express DNA sequencer (Pharmacia) using ThermoSequenase as recommended by the manufacturer (Amersham). The nucleotide sequences were compared to entries in the database at the National Center for Biotechnology Information using the program BLASTN (Altschul *et al.*, 1990). The phylogeny of the sequences was analysed by parsimony using the program DNAPARS of the PHYLIP 3.5c package (Felsenstein, 1995) and by calculating a distance matrix according to the two-parameter model of Kimura (1980) followed by the construction of a phylogenetic tree using the neighbour-joining method (Saitou & Nei, 1987) as implemented in the program TREECON (Van de Peer & De Wachter, 1995).

RESULTS

Acidaminobacter hydrogenoformans belongs to clostridial cluster XI

Although *Acidaminobacter hydrogenoformans* stains Gram-negative, its ultrastructure suggests that it belongs to the Gram-positive bacteria (Stams & Hansen, 1984). In order to clarify the phylogenetic

Fig. 1. Phylogenetic relationships of strain glu 65 (*Acidaminobacter hydrogenoformans*) with species belonging to the low-G+C Gram-positive bacteria. The phylogenetic tree is based on a distance matrix of 16S rRNA sequences and was constructed using the neighbour-joining method. *Amycolatopsis methanolica* was used as an outgroup. Bootstrap values (percentage) are based on 500 replicates and are given at each node. The accession numbers of the sequences of the reference strains are given in parentheses.

Fig. 2. Phylogenetic relationships of strain asp 66^T (*Propionibacter pelophilus*) with species of the β -subclass of the *Proteobacteria*. The phylogenetic tree is based on a distance matrix of 16S rRNA sequences and was constructed using the neighbour-joining method. *Escherichia coli* was used as an outgroup. Bootstrap values (in percentages) are based on 500 replicates and are given at each internal node. The accession numbers of the sequences of the reference strains are given in parentheses.

position of this strain, the 16S rRNA gene was amplified by PCR and the nucleotide sequence was subsequently determined. Comparison of the nucleotide sequence (1468 bp) of the rRNA gene of *Acidaminobacter hydrogenoformans* with entries in GenBank showed that it was most similar to 16S rRNA genes of species belonging to the low-G+C sub-group of the Gram-positive bacteria. The phylogenetic analysis of *Acidaminobacter hydrogenoformans* consistently (100% bootstraps) placed this bacterium in the phenotypically diverse cluster XI of the *Clostridium* subphylum (Fig. 1). *Acidaminobacter hydrogenoformans* displayed only a loose association (sequence similarity levels between 81 and 84%) with *Eubacterium saphenum*, *Eubacterium brachy*, *Eubacterium timidum* and 'Clostridium aminobutyricum' and grouped with *Clostridium halophilum* but with a rather low bootstrap value.

Strain asp 66^T belongs to the β -subclass of the *Proteobacteria*

The 16S rRNA gene from strain asp 66^T was amplified by PCR and the nucleotide sequence of the PCR product was determined directly. The sequence consisted of 1485 nucleotides, which is approximately 97% of the complete 16S rRNA gene. A comparison

of the asp 66^T rRNA gene with entries in GenBank showed that this gene was highly similar to 16S rRNA genes of bacteria belonging to the β -subclass of the *Proteobacteria*, in particular to *Rhodocyclus tenuis* (94% similarity), *Rhodocyclus purpureus* (93% similarity) and *Azoarcus* sp. strain 6a3 (94.5% similarity). This was confirmed by a phylogenetic analysis in which asp 66^T consistently clustered with the *Rhodocyclus* species (Fig. 2, 100% bootstraps). A maximum-parsimony analysis using DNAPARS resulted in one most-parsimonious tree with the same topology as the distance tree shown in Fig. 2.

DISCUSSION

A phylogenetic analysis of *Acidaminobacter hydrogenoformans* based on the nucleotide sequence of the 16S rRNA gene showed it to be a member of the *Clostridium* subphylum of the Gram-positive bacteria. This grouping with Gram-positives is in agreement with electron micrographs of the cell wall in which an outer membrane was not visible (Stams & Hansen, 1984). *Acidaminobacter hydrogenoformans* displays some similarities with *Eubacterium acidaminophilum* (Zindel *et al.*, 1988). Both organisms are strict anaerobes that can grow on glycine and on serine in pure culture and utilize more amino acids such as

Table 1. Comparison of *Pelobacter propionicus* with other important bacteria with a propionic fermentation and with its phylogenetic neighbours

Organism(s)	Phylogenetic group	Sugars fermented	Oxygen tolerance	N ₂ fixation	Dissimilatory sulfate reduction	Propionic fermentation
<i>Propionibacter pelophilus</i>	β -Proteobacteria	Yes	Rather oxygen-tolerant anaerobe	Yes	No	Yes
<i>Pelobacter propionicus</i>	δ -Proteobacteria	No	Obligate anaerobe	Yes	No	Yes
<i>Desulfobulbus propionicus</i>	δ -Proteobacteria	No	Obligate anaerobe	Yes	Yes	Yes
<i>Propionibacterium</i>	High-G + C Gram-positives	Yes	Oxygen-tolerant anaerobe	No	No	Yes
<i>Rhodocyclus tenuis</i>	β -Proteobacteria	No	Anoxygenic phototroph, facultative anaerobe	Yes	No	No
<i>Azoarcus</i>	β -Proteobacteria	No	Aerobe or facultative anaerobe	Yes (most strains)	No	No

alanine and leucine when grown in co-culture with a hydrogen and formate-utilizing methanogen or *Desulfovibrio* strain. The substrate spectrum, however, differs considerably. *Eubacterium acidaminophilum* also stains Gram-negative but has a Gram-positive cell wall structure. Syntrophic utilization of amino acids (alanine and isoleucine) has also been reported for *Clostridium sporogenes*, an organism that in pure culture can dispose of reducing equivalents in a Stickland reaction (Wildenauer & Winter, 1986; Winter *et al.*, 1987). *C. sporogenes* is a member of cluster I of the *Clostridium* subphylum (Collins *et al.*, 1994). Cluster XI is only distantly related to the true clostridia of cluster I and according to Collins *et al.* (1994) will comprise a number of new genera after a complete revision of the genus *Clostridium*. In the phylogenetic tree of Fig. 1 strain glu 65 grouped with *Clostridium halophilum*, an organism with a G + C content of over 20 mol % lower than strain glu 65; this species utilizes a number of amino acids in a Stickland reaction with betaine and also ferments several sugars (Fendrich *et al.*, 1990). The phylogenetic position of glu 65 in cluster XI is such that it clearly constitutes a separate genus and its status as the type strain of *Acidaminobacter hydrogeniformans* therefore requires no changes.

Strain asp 66^T belongs to the β -subclass of the *Proteobacteria* with isolate 6a3 of Reinhold-Hurek *et al.* (1993) and *R. tenuis* as its closest relatives (94.5 and 94% similarity in 16 S rRNA gene sequence, respectively). Strain 6a3 belongs to *Azoarcus* cluster D. The genus *Azoarcus* is known to consist of a number of phylogenetically distantly related clusters (Hurek *et al.*, 1997) of nitrogen-fixing organisms with a respiratory metabolism. *R. tenuis* is an anoxygenic phototroph; it utilizes a wide variety of substrates, can fix N₂ and in the dark it can grow with oxygen as electron acceptor (Trüper & Imhoff, 1992). The phylogenetic and phenotypic differences with related taxa (Table 1) indicate that strain asp 66^T belongs to a new genus and species for which we propose the name *Propionibacter pelophilus*. The description (see below) is based on data from the present study and from Hansen *et al.* (1990).

Propionibacter pelophilus is the first representative of the β -subclass of the *Proteobacteria* with a propionic fermentation. A propionic fermentation is carried out

by a wide variety of bacteria belonging to various phylogenetic groups. Examples are the high-G + C Gram-positive genus *Propionibacterium* (Olsen *et al.*, 1994), some representatives of the *Clostridium* subphylum (see Tholozan *et al.*, 1992; Evers *et al.*, 1993; Collins *et al.*, 1994, for example), certain rod-shaped species of the δ -subclass of the *Proteobacteria* (Schink, 1984), coccoid and curved bacteria which belong to cluster IX of the Gram-positive anaerobes such as *Veillonella*, *Selenomonas* and *Pectinatus* (Collins *et al.*, 1994; Willems & Collins, 1995), and Gram-negative vibrioid or spirilloid bacteria of unknown phylogenetic position such as *Anaerovibrio* (Prins, 1984) and *Propionispira* (Schink *et al.*, 1982). A propionic fermentation with succinate as an additional product was recently reported for oxygen-tolerant saccharolytic ultramicrobacteria from rice paddy soil belonging to the *Verrucomicrobiales* (Janssen *et al.*, 1997). Among the *Proteobacteria*, bacteria that can carry out a propionic fermentation have been found in two genera of the δ -subclass, *Pelobacter* and *Desulfobulbus*. *Desulfobulbus propionicus* is a rod-shaped, sulfate-reducing bacterium which, in the absence of sulfate, ferments some substrates (lactate, pyruvate and ethanol) to acetate and propionate via a succinate pathway that involves transcarboxylase (Stams *et al.*, 1984). The strict anaerobe *Pelobacter propionicus* carries out a propionic fermentation with only a few substrates not including sugars. Despite similarities such as the ability to fix N₂, the propionic fermentation, cellular morphology and only a small difference in the G + C content of the DNA, *Propionibacter pelophilus* and *Pelobacter propionicus* appear to belong to different phylogenetic sub-groups (Evers *et al.*, 1993; Schink, 1984).

Description of *Propionibacter* gen. nov.

Propionibacter (Pro.pioni.ba'cter. M.L. n. *acidum propionicum* propionic acid; M.L. masc. n. *bacter* equivalent of Gr. neut. n. *bakterion* small rod; M.L. masc. n. *Propionibacter* propionic acid rod).

Gram-negative rod-shaped cells that may be motile by one polar flagellum. Multiplying by binary fission. No spores formed. Anaerobic to aerotolerant metabolism. In the absence of nitrate substrates are fermented with

propionate and acetate as characteristic products. Growth is supported by simple organic compounds such as sugars, dicarboxylic acids, sugar alcohols and aspartate. Nitrate reduced to nitrite. Does not use sulfate as electron acceptor. Can utilize molecular nitrogen as nitrogen source. The genus belongs to the β -subclass of the *Proteobacteria*. Type species *Propionibacter pelophilus*.

Description of *Propionibacter pelophilus* sp. nov.

Propionibacter pelophilus (pe.lo'phi.lus. Gr. n. *pelos* mud; Gr. adj. *philus* loving; M.L. adj. *pelophilus* mud-loving).

Cells are rod-shaped (0.9–1.1 μ m long and 0.5–0.6 μ m wide). Gram-negative cell envelope ultrastructure with an outer membrane. Cells may be motile by means of one polar flagellum. Colonies on anaerobic plates with aspartate as substrate are slimy white. Grows in mineral media with a single fermentable substrate. Uses the following energy substrates: L-aspartate, fumarate, L-malate, pyruvate, oxaloacetate, citrate, fructose, glucose, xylose, gluconate, arabitol, xylitol, mannitol, arabinose and mannose. Does not ferment glycine, L-alanine, L-serine, L-threonine, L-methionine, L-glutamate, L-histidine, L-arginine, L-lysine, lactate, α -ketoglutarate, glycolate, succinate, propionate, butyrate, glycerol, ethanol, propanol, 2,3-butanediol, acetoin, cellulose, cellobiose, lactose, sucrose, maltose, melibiose, starch, sorbose, rhamnose, galactose, sorbitol, pectin, peptone, xylan or inulin. Indole-negative, urease-negative; does not liquefy gelatin. Growth on glucose and aspartate possible at initial pH of 6.5–8.5, the optimum being around 7.5–8.0. Final pH of glucose-fermenting cultures is 5.5. Temperature optimum 27–30 $^{\circ}$ C; no growth at 35 $^{\circ}$ C. Sulfate can be used as sulfur source but growth in the absence of sulfide leads to cell clumps. Best growth under anoxic conditions in sulfide-reduced media but growth is possible in stationary cultures under air. No growth in well-aerated cultures. G+C content of the DNA is 60.8 mol%. Habitat: anoxic freshwater and estuarine sediments. Type strain: strain asp 66^T (= DSM 12018^T).

ACKNOWLEDGEMENTS

We thank Peter Terpstra for excellent technical assistance.

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