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Propionivibrio limicola sp. nov., a fermentative bacterium specialized in the degradation of hydroaromatic compounds, reclassification of *Propionibacter pelophilus* as *Propionivibrio pelophilus* comb. nov. and amended description of the genus *Propionivibrio*

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Strain GolChi1^T, a mesophilic, anaerobic bacterium, was isolated with quinic acid (1,3,4,5-tetrahydroxy-cyclohexane-1-carboxylic acid) as the sole source of carbon and energy. Of more than 30 substrates tested, only the hydroaromatic compounds quinic acid and shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) were utilized, yielding acetate and propionate as the only fermentation products. Sugars, alcohols, (di-)carboxylic acids, amino acids and aromatic compounds were not fermented and no external electron acceptors were used. Strain GolChi1^T is a Gram-negative, rod-shaped, aerotolerant anaerobe that possesses superoxide dismutase; it does not employ the classical hydroaromatic pathway of aerobic bacteria for the degradation of hydroaromatic compounds (no aromatic intermediates involved). 16S-rRNA-based phylogenetic analyses revealed a common origin of this isolate and *Rhodocyclus*, *Propionibacter* and *Propionivibrio* species. High sequence similarity (> 96%) and phenotypic traits indicated a closer relationship between strain GolChi1^T and the type species of the monospecific genera *Propionivibrio* and *Propionibacter* but, due to its phenotypic properties, strain GolChi1^T could not be assigned conclusively to either of these taxa. We propose (i) the amended description of the genus *Propionivibrio*, (ii) the reclassification of *Propionibacter pelophilus* Meijer *et al.* 1999 as *Propionivibrio pelophilus* comb. nov. and (iii) designation of *Propionivibrio limicola* sp. nov., with the type strain GolChi1^T (= DSM 6832^T = ATCC BAA-290^T).

Keywords: anaerobic degradation, fermentation, hydroaromatic compounds, quinic acid, shikimic acid

The biosynthesis of aromatic compounds via the shikimic acid pathway involves hydroaromatic compounds as important intermediates (Herbert, 1981). Quinic acid and shikimic acid, which are important precursors of lignin and tannin biosynthesis, are stored in considerable amounts in the vacuoles of many vascular plants (Yoshida *et al.*, 1975). The degradation of hydroaromatic compounds by aerobic bacteria and fungi proceeds oxidatively via the hydroaromatic pathway, involving aromatic intermediates (for references, see Brune & Schink, 1992).

Fermentative degradation of hydroaromatic compounds by anaerobic bacteria has been shown only in the past decade. Several strains have been enriched and isolated from marine and freshwater sediments with quinic acid as the sole source of carbon and energy (Brune & Schink, 1992). Two of these isolates, the marine strain VenChi2^T and the freshwater strain GolChi1^T, have been characterized morphologically

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and physiologically in detail. Both strains degrade hydroaromatic compounds via novel, fermentative pathways that do not involve aromatic intermediates (Brune & Schink, 1992). The unique phenotypic traits of the two strains, however, did not point to a taxonomic affiliation. Here, we present the results of the phylogenetic analysis of strain GolChi1^T, together with additional phenotypic data, and propose the designation of a novel species in the genus *Propionivibrio*. The results of the phylogenetic analysis of strain VenChi2^T have been presented elsewhere (Brune *et al.*, 2002).

Characterization of strain GolChi1^T

Pure cultures of strain GolChi1^T, which was originally isolated from freshwater sediment of a eutrophic pond near Tübingen, Germany, were taken from our laboratory collection. Cells were routinely cultivated in oxygen-free, bicarbonate-buffered mineral medium with 5 mM sodium quinate (1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid, sodium salt) as the sole source of carbon and energy. Details are given in the original description (Brune & Schink, 1992).

Strain GolChi1^T is restricted to the fermentation of hydroaromatic substrates. More than 30 different substrates were tested, and only quinic acid and shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) were utilized. Sugars (cellobiose, fructose, glucose, erythrose, lactose, ribose, xylose), alcohols (*meso*-erythritol, ethanol, glycerol, mannitol), carboxylic acids (citrate, crotonate, fumarate, glycolate, 2-hydroxybutyrate, 3-hydroxybutyrate, 4-hydroxybutyrate, lactate, malate, 2-oxobutyrate, pyruvate, sorbate, tartrate), amino acids (alanine, aspartate, glycine, threonine) and aromatic compounds (gallate, phloroglucinol, protocatechuate, resorcinol, 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxycinnamate) were not fermented (Brune & Schink, 1992). External electron acceptors (amorphous ferric iron, nitrate, oxygen, sulfate, sulfur, thiosulfate) were not reduced with lactate, propionate or quinate as the electron donor.

Additional growths tests on D-glucose (5 mM) and disodium L-malate, disodium fumarate and sodium L-lactate (each 10 mM) performed in medium supplemented with L-phenylalanine, L-tyrosine and L-tryptophan (each 50 μM) were negative. This indicates that the absence of growth on these compounds is not caused by an inability of strain GolChi1^T to synthesize aromatic amino acids in the absence of quinate or shikimate as precursors. Other physiological properties of strain GolChi1^T have been documented in detail (Brune & Schink, 1992); taxonomically relevant traits are summarized in the species description.

Phylogenetic analysis

16S-rRNA-encoding DNA fragments were amplified *in vitro* and sequenced directly as described earlier (Springer *et al.*, 1992; Ludwig *et al.*, 1998). Using the automated tools of the ARB software package (Ludwig & Strunk, 1996), the new 16S rRNA sequences were fitted into an alignment of about 22000 homologous full and partial primary structures available in public databases (Ludwig, 1995). Distance-matrix, maximum-parsimony and maximum-likelihood methods were applied as implemented in the ARB software package. Different datasets were analysed, varying with respect to the sequences of outgroup reference organisms included and alignment positions selected according to their degrees of conservation.

Phylogenetic tree analysis showed that strain GolChi1^T represents a phylogenetic subgroup of the *β*-subclass of the *Proteobacteria* that comprises *Rhodocyclus*, *Propionibacter* and *Propionivibrio* species. Strain GolChi1^T shares the highest 16S rRNA sequence similarity (93·3–96·4 %) with species of the genera *Ferribacterium* (Cummings *et al.*, 1999), *Dechloromonas* (Achenbach *et al.*, 2001), *Rhodocyclus* (Dewhirst *et al.*, 1990), *Propionivibrio* (Hippe *et al.*, 1999) and *Propionibacter* (Meijer *et al.*, 1999) (Fig. 1). In comprehensive phylogenetic trees, this group is placed in the neighbourhood of *Azoarcus*, *Thauera*, *Hydrogenophilus* and *Zoogloea* (represented by *Azoarcus evansii* and *Thauera aromatica* in Fig. 1; Anders *et al.*, 1995) in the *β*-subclass of the *Proteobacteria*.

A closer relationship of strain GolChi1^T to *Propionibacter pelophilus* and *Propionivibrio dicarboxylicus* (96·4 and 96·0 % sequence similarity, respectively) was supported by all treeing analyses performed. Also, the DNA G+C content of strain GolChi1^T (61·6 mol %) is very similar to the values reported for *Propionibacter pelophilus* (60·8 mol %; Meijer *et al.*, 1999) and *Propionivibrio dicarboxylicus* (61 mol %; Tanaka *et al.*, 1990), whereas it differs slightly from the narrow range of values spanned by members of the genus *Rhodocyclus* (64·8–65·3 mol %; Trüper & Imhoff, 1992).

Taxonomic considerations

In addition to the high 16S rRNA gene sequence similarity, strain GolChi1^T, *Propionivibrio dicarboxylicus* and *Propionibacter pelophilus* also share a number of phenotypic traits that separate them clearly from their closest phylogenetic relatives. All three strains are chemotrophic organisms with a fermentative metabolism and form propionate and acetate as the major products, which allows their classification as the only members of the *β-Proteobacteria* that perform a propionic acid fermentation. All three form rod-shaped cells that are motile by a single polar flagellum. Strain GolChi1^T and *Propionibacter pelophilus* are both aerotolerant.

Nevertheless, strain GolChi1^T is clearly separated from the existing species. *Propionivibrio dicarboxylicus* is a curved rod, utilizes maleate, fumarate and L-malate and decarboxylates succinate to propionate, whereas *Propionibacter pelophilus* ferments simple organic compounds (sugars, dicarboxylic acids, sugar

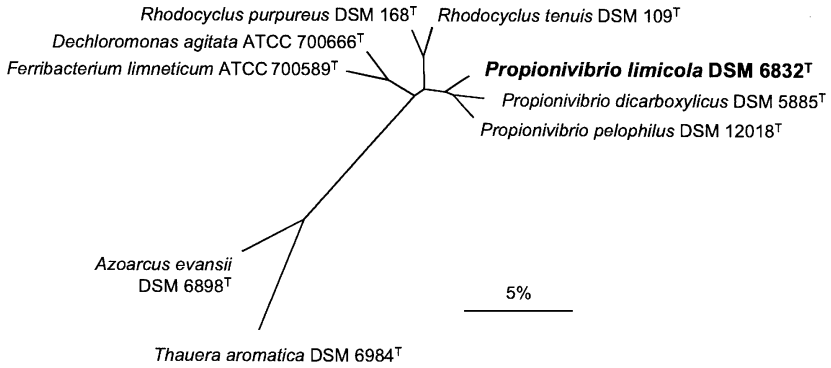

Fig. 1. Phylogenetic tree (16S rRNA) showing the position of *Propionivibrio limicola* strain GolChi1^T, *Propionivibrio dicarboxylicus* and *Propionivibrio* [*Propionibacter*] *pelophilus* among related taxa of the β-subclass of the *Proteobacteria*. The tree was reconstructed and optimized using the maximum-parsimony tool implemented in the ARB package (Ludwig & Strunk, 1996), including all available sequence that were at least 90% complete (in comparison with the *Escherichia coli* sequences). Only alignment positions sharing identical residues in at least 50% of all representatives of the subclass were included. The tree topology was evaluated and corrected according to the results obtained by applying distance and maximum-likelihood approaches. Only type strains are shown. Accession numbers are: *Dechloromonas agitata* ATCC 700666^T, AF047462; *Ferribacterium limneticum* ATCC 700589^T, Y17060; *Rhodocyclus purpureus* DSM 168^T, M34132; *Rhodocyclus tenuis* DSM 109^T, D16208; *Propionivibrio limicola* DSM 6832^T, AJ307983; *Propionivibrio dicarboxylicus* DSM 5885^T, Y17601; *Propionivibrio pelophilus* DSM 12018^T, AF016690; *Azoarcus evansii* DSM 6898^T, X77679; *Thauera aromatica* DSM 6984^T, X77118. Bar, 5% estimated sequence divergence.

alcohols) and reduces nitrate. None of these traits is present in strain GolChi1^T. Interestingly, growth tests performed with *Propionibacter pelophilus* DSM 12018^T revealed that this organism is also capable of fermenting quinic acid and shikimic acid, forming acetate and propionate as the major products (data not shown). Nevertheless, since strain GolChi1^T can be distinguished clearly from the existing species not only by its unique metabolism but also by phylogenetic distance (Stackebrandt & Goebel, 1994), it should be assigned to a separate species.

The genus *Propionivibrio* was described by Tanaka *et al*. (1990) and contains a single species, *Propionivibrio dicarboxylicus*. The phylogenetic position of *Propionivibrio dicarboxylicus* (Hippe *et al*., 1999) was published almost simultaneously with the description of the genus *Propionibacter*, which contains *Propionibacter pelophilus* as its only species (Meijer *et al*., 1999). The small differences in the 16S rRNA gene sequences of these species and their similar fermentation patterns have prompted Hansen (2002) to suggest the inclusion of *Propionibacter pelophilus* in the genus *Propionivibrio*, an argument that would also apply to the taxonomic affiliation of strain GolChi1^T. However, the original genus description of *Propionivibrio* is rather narrow and contains references to phenotypic traits such as cell curvature, substrates and an exact DNA base ratio, which should really be reserved for a species level description. Instead of creating yet another monospecific genus, we prefer to take up the suggestion of Hansen (2002), who has already recommended the amendation of the description of the genus *Propionivibrio* to allow the inclusion of *Propionibacter pelophilus*, and propose to allocate all three species to the genus *Propionivibrio*.

Amended description of *Propionivibrio* Tanaka *et al.* 1990 emend.

Gram-negative rods (straight or curved). Do not form spores. May be motile by means of a single polar flagellum. Multiply by binary fission. Chemo-organotrophic metabolism. Substrates are fermented to propionate and acetate as major products. Strictly anaerobic to aerotolerant. Some species may use external electron acceptors. Based on their 16S rRNA sequences, members of this genus form a monophyletic group within the β-subclass of the *Proteobacteria*. The type species is *Propionivibrio dicarboxylicus* Tanaka *et al*. 1990.

Description of *Propionivibrio limicola* sp. nov.

Propionivibrio limicola (li.mi′co.la. L. n. *limus* mud; L. v. *colere* to inhabit; N.L. adj. *limicola* living in mud).

Cells are straight, slender rods, 0·6–0·7 μm wide and 1·5–2·5 μm long. Cells are motile (polar monotrichously flagellated), Gram-negative, oxidase-negative, catalase-negative, superoxide-dismutase-positive. No spores are formed. Chemo-organotrophic, fermentative metabolism; external electron acceptors are not used. Contains no cytochromes. Quinic acid and shikimic acid are the only substrates, which are fermented to acetate, propionate and CO_2 as the only products. No growth with sugars (cellobiose, fructose, glucose, erythrose, lactose, ribose, xylose), alcohols (*meso*-erythritol, ethanol, glycerol, mannitol),

boxylic acids (citrate, crotonate, fumarate, glycolate, 2-hydroxybutyrate, 3-hydroxybutyrate, 4-hydroxybutyrate, lactate, malate, 2-oxobutyrate, pyruvate, sorbate, tartrate), amino acids (alanine, aspartate, glycine, threonine) or aromatic compounds (gallate, phloroglucinol, protocatechuate, resorcinol, 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxycinnamate). External electron acceptors (amorphous ferric iron, nitrate, oxygen, sulfate, sulfur, thiosulfate) are not used. Aerotolerant; growth occurs in non-reduced media when incubated under air without agitation. pH range for growth is 6·0–8·0, with an optimum around pH 7·0–7·5. Temperature optimum is 37 °C; no growth at 45 °C. Optimal growth in freshwater medium (identical growth rates with quinic acid and shikimic acid: $\mu = 0{\cdot}22\ h^{-1}$). Growth is inhibited completely in brackish medium with 10 g NaCl and 1·0 g $MgCl_2\ l^{-1}$.

DNA base ratio: $61{\cdot}6 \pm 0{\cdot}2$ mol% G+C. Habitat: anoxic freshwater sediment. Type strain: GolChi1^T (= DSM 6832^T = ATCC BAA-290^T).

Description of *Propionivibrio pelophilus* comb. nov.

Basonym: *Propionibacter pelophilus* Meijer *et al.* 1999.

The genus *Propionibacter* was described by Meijer *et al.* (1999) to harbour the newly described species *Propionibacter pelophilus*. Following the suggestion of Hansen (2002), we propose to reclassify *Propionibacter pelophilus* as *Propionivibrio pelophilus* comb. nov. after emendation of the description of *Propionivibrio*. By this transfer, the genus *Propionibacter* loses its only species and becomes void. The traits 'nitrate reduced to nitrite' and 'utilizes N_2 as nitrogen source', formerly included in the description of *Propionibacter* (Meijer *et al.*, 1999), are added to the description of *Propionivibrio pelophilus*.

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