

Ilyobacter insuetus sp. nov., a fermentative bacterium specialized in the degradation of hydroaromatic compounds

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The mesophilic, anaerobic bacterium strain VenChi2^T 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 quinic acid and shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) were utilized, yielding acetate, propionate, butyrate, H₂ and CO₂ as fermentation products. Sugars, alcohols, (di-)carboxylic acids, amino acids and aromatic compounds were not fermented and no external electron acceptors were used. Strain VenChi2^T is a Gram-negative, strictly anaerobic, coccoid rod; it does not employ the classical hydroaromatic pathway of aerobic bacteria for the degradation of hydroaromatic compounds (no aromatic intermediates involved). Comparative 16S and 23S rDNA sequence analyses placed strain VenChi2^T in the fusobacteria phylum, with the closest relatives among species of the genera *Ilyobacter* and *Propionigenium*. The results indicate that, disregarding the taxonomically misplaced *Ilyobacter delafieldii*, which is a member of the clostridia, the validly described *Ilyobacter* and *Propionigenium* species are phylogenetically intermixed. Based on its phenotypic properties, strain VenChi2^T (= DSM 6831^T = ATCC BAA-291^T) is assigned to the genus *Ilyobacter* as the type strain of a novel species, *Ilyobacter insuetus* sp. nov.

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

Hydroaromatic compounds are important intermediates in the biosynthesis of aromatic compounds via the shikimic acid pathway (Herbert, 1981). As precursors of lignin and tannin biosynthesis, quinic acid and shikimic acid are stored in considerable amounts in the vacuoles of many vascular plants (Yoshida *et al.*, 1975). Aerobic bacteria and fungi degrade hydroaromatic compounds oxidatively via the hydroaromatic pathway, involving aromatic intermediates (for references, see Brune & Schink, 1992).

The anaerobic degradation of hydroaromatic compounds by fermentative 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. A detailed morphological and physiological characterization of two of these isolates, the marine strain VenChi2^T and the freshwater strain GolChi1, has been published (Brune & Schink, 1992). Both strains were found to degrade hydroaromatic compounds via novel, fermentative pathways that do not involve aromatic intermediates (Brune & Schink, 1992). However, the unique phenotypic traits of the two strains did not allow the determination of their taxonomic affiliation. In the present paper, we present the results of a phylogenetic analysis by 16S and 23S rRNA gene sequence comparison for strain VenChi2^T together with additional phenotypic data and propose the designation of a novel species in the genus *Ilyobacter*. The results of the phylogenetic analysis of strain GolChi1 will be presented elsewhere (Brune *et al.*, 2002).

Published online ahead of print on 14 December 2001 as DOI 10.1099/ijs.0.01847-0.

The GenBank/EMBL accession numbers for the 16S and 23S rRNA gene sequences of strain VenChi2^T are respectively AJ307980 and AJ307976.

Characterization of strain VenChi2^T

Pure cultures of strain VenChi2^T (= DSM 6831^T), which was originally isolated from organic-rich marine sediment from the canals of Venice, Italy, were taken from our laboratory collection. Cultivation was performed routinely in oxygen-free, bicarbonate-buffered mineral medium with 5 mM sodium quinate (1,3,4,5-tetrahydroxy-cyclohexane-1-carboxylic acid, sodium salt) as the sole source of carbon and energy. Details are given in the original description (Brune & Schink, 1992).

Strain VenChi2^T is restricted to the fermentation of hydroaromatic substrates. Of more than 30 different substrates tested, 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 electron donors.

Additional growth tests on D-glucose (5 mM), 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 simply by an inability of strain VenChi2^T to synthesize aromatic amino acids in the absence of quinate and shikimate as precursors. Other physiological properties of strain VenChi2^T have been documented in detail (Brune & Schink, 1992); taxonomically relevant traits are summarized in the species description (see below).

Phylogenetic analysis

16S- and 23S-rRNA-encoding DNA fragments were amplified *in vitro* and sequenced directly as described previously (Springer *et al.*, 1992; Ludwig *et al.*, 1992, 1995). The new sequences were fitted into alignments of about 22000 (16S rRNA) and 4000 (23S rRNA) homologous full and partial primary structures available in public databases (Ludwig, 1995) using the respective automated tools of the ARB software package (Ludwig & Strunk, 1996). 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 to alignment positions selected according to their degree of conservation.

Phylogenetic treeing placed strain VenChi2^T among the fusobacteria phylum. In this phylum, three major clusters can be differentiated according to the results of comparative 16S rDNA sequence analysis obtained to date (Ludwig *et al.*, 1998): the *Sebaldeella*-*Streptobacillus*-*Leptotrichia* lineage, the *Fusobacterium* branch and the *Ilyobacter*-*Propionigenium* group (Fig. 1). Depending on the method and parameters of treeing, *Fusobacterium perfoetens* and *Cetobacterium cetens* may cluster with the fusobacteria or may have an intermediate status between the fusobacteria and the *Ilyobacter*-*Propionigenium* group (not shown in Fig. 1). Within the latter group, a separate status of *Propionigenium maris* (Janssen & Liesack, 1995) was indicated in the majority of 16S-rRNA-based analyses and is supported by position and/or branch lengths in trees (Fig. 1) as well as by lower overall rRNA sequence similarities. The *Ilyobacter* species, strain VenChi2^T and *Propionigenium modestum* (Both *et al.*, 1991) share 97.4–98.5% 16S and 96.8–98.5% 23S rRNA sequence identity, whereas the corresponding values for *Propionigenium maris* are 96.5–96.8% and 94.4–94.9%. A stable relative branching order within the *Ilyobacter*-*Propionigenium* cluster was supported by the 23S-rRNA-based phylogenetic analyses applying alternative treeing approaches, but could not be obtained from comparative 16S rRNA analyses. The range of overall 16S rRNA sequence similarities of 96.6–97.6% shared by strain VenChi2^T and the other members of the *Ilyobacter*-*Propionigenium* group includes the threshold value for species separation (Stackebrandt & Goebel, 1994).

Taxonomic considerations

The placement of strain VenChi2^T in a separate species is supported by its DNA base composition (35.7 mol% G + C, determined by HPLC), which is slightly higher than the values reported for *Ilyobacter tartaricus* and *Ilyobacter polytropus* (33.1 and 32.2 mol% G + C, determined by thermal denaturation; Schink, 1984; Stieb & Schink, 1984) and also differs from the values reported for *Propionigenium modestum* and *Propionigenium maris* (33.9 and 40 mol% G + C; Schink & Pfennig, 1982; Janssen & Liesack, 1995).

The strongest argument for allocating strain VenChi2^T to a separate species, however, is its unusual phenotype, i.e. the metabolic restriction to hydroaromatic compounds as carbon and energy sources. Both species in the genus *Propionigenium* are characterized by their ability to grow by fermentation of dicarboxylic acids to propionate and acetate and by decarboxylation of succinate to propionate (Schink, 1992; Janssen & Liesack, 1995). These traits are absent in strain VenChi2^T. *Propionigenium maris* has greater metabolic versatility than the type species, *Propionigenium modestum*, and shares with both species in the genus *Ilyobacter* the ability to ferment various organic acids and several carbohydrates to lactate, acetate, formate, ethanol or butyrate (Schink, 1984; Stieb & Schink, 1984; Janssen & Liesack, 1995). None of these sub-

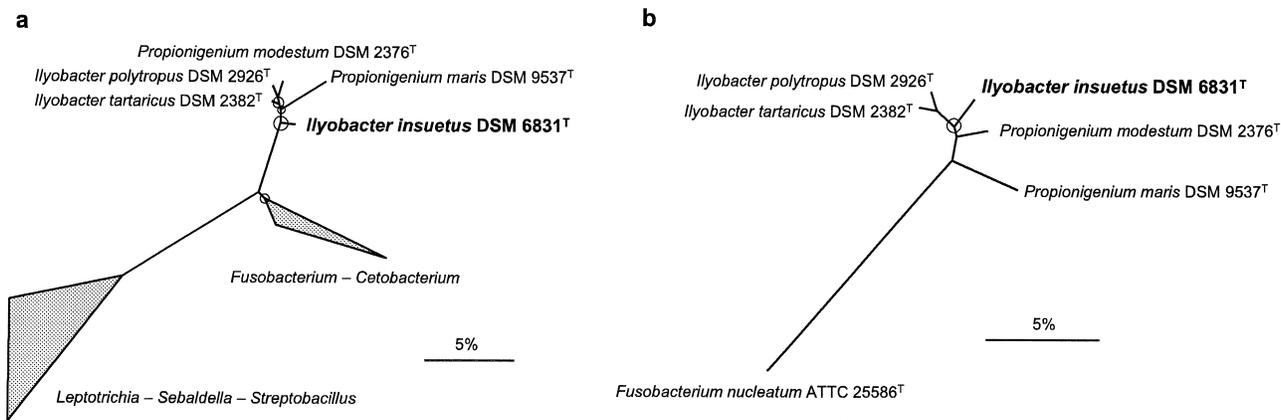


Fig. 1. 16S- (a) and 23S- (b) rRNA-based trees reflecting the phylogenetic relationships of *Ilyobacter insuetus* VenChi2^T within the radiation of representatives of the fusobacteria phylum. The trees were reconstructed and optimized using the ARB-implemented maximum-parsimony tool including all sequences available that were $\geq 90\%$ complete (in comparison with the *Escherichia coli* sequences). Only alignment positions sharing identical residues in at least 50% of all representatives of the phylum were included. The low significance of the internal structure of the *Ilyobacter*–*Propionigenium* cluster is indicated by ranges of uncertainty shown by circles, which were estimated by an ‘upper bootstrap limit’ tool of the ARB package (Ludwig & Strunk, 1996). The topologies of the trees were evaluated and corrected according to the results obtained by applying distance and maximum-likelihood approaches. In the case of 23S rRNA, the topology shown was supported when the alternative treeing methods (distance and maximum-likelihood methods) were applied. In the case of 16S rRNA, however, no common internal branching order could be found for the cluster. Triangles indicate phylogenetic groups. Only type strains are shown. The strain designations and GenBank/EMBL accession numbers of the 16S and 23S rRNA sequences are: *I. tartaricus* GraTa2^T (= DSM 2382^T), AJ307982 (16S), AJ307977 (23S); *I. polytropus* CuHbu1^T (= DSM 2926^T), AJ307981, AJ307975; *I. insuetus* VenChi2^T (= DSM 6831^T = ATCC BAA-291^T), AJ307980, AJ307976; *P. modestum* GraSucc2^T (= DSM 2376^T), X54275, AJ307978; *P. maris* 10succ1^T (= DSM 9537^T), X84049, AJ307979; *Fusobacterium nucleatum* ATCC 25586^T (= DSM 20482^T), M58683, AJ307974. Bar, 5% estimated sequence divergence.

strates supported the growth of strain VenChi2^T. Butyrate and H₂ formation, which are characteristics of strain VenChi2^T, are found only with *Ilyobacter polytropus* (Stieb & Schink, 1984) and *Propionigenium maris* (Janssen & Liesack, 1995).

The results of the comparative rRNA gene sequence analyses clearly indicate a monophyletic and separate status of the group formed by *Ilyobacter polytropus*, *Ilyobacter tartaricus*, *Propionigenium modestum*, *Propionigenium maris* and strain VenChi2^T (Fig. 1). With the present taxonomic placements, the members of different genera are phylogenetically intermixed and taxonomic revision will be necessary. Given the high overall sequence similarities within this group (96.5% for 16S rRNA and 94.4% for 23S rRNA), it might be possible to unite the species in the *Propionigenium*–*Ilyobacter* group in a common genus (Ludwig *et al.*, 1998). In that case, according to the rules of nomenclature (Lapage *et al.*, 1992), the genus *Propionigenium*, validly published in 1982 (Schink & Pfennig, 1982), would have priority over *Ilyobacter* (Stieb & Schink, 1984). However, the considerable phenotypic differences between the existing species in the *Ilyobacter*–*Propionigenium* group and their largely unexplored metabolic diversity appear counter-indicative of such a union and would rather call for additional genera to be created.

At present, we consider it premature to revise this group – any such action should be postponed until

more information is available. Since the genus *Propionigenium* is reserved explicitly for bacteria that form propionate as the main fermentation product (Schink & Pfennig, 1982), whereas the description of the genus *Ilyobacter* contains no restriction with respect to the fermentation products formed (Stieb & Schink, 1984), and since strain VenChi2^T shares with the described *Ilyobacter* species the inability to grow by decarboxylation of succinate to propionate, which separates them from the described *Propionigenium* species, we propose to assign strain VenChi2^T to the genus *Ilyobacter* as *Ilyobacter insuetus* sp. nov.

Description of *Ilyobacter insuetus* sp. nov.

Ilyobacter insuetus (in.su.e'tus. L. masc. adj. *insuetus* unusual, extraordinary, referring to the organism's metabolism).

Coccoid cells, 1.0–1.5 μm long and 0.8–1.0 μm wide, non-motile, Gram-negative, oxidase-negative, catalase-negative, superoxide-dismutase-negative. Oxygen-sensitive; no growth under air, but growth in non-reduced media. 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, butyrate, hydrogen and CO₂. No growth with 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) or aromatic compounds (gallate, phloroglucinol, protocatechuate, resorcinol, 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxycinnamate). No external electron acceptors (amorphous ferric iron, nitrate, oxygen, sulfate, sulfur, thiosulfate) are used. Strict anaerobe, growth only in reduced medium. pH range for growth 6.0–9.0, broad optimum around pH 6.5–8.0. Temperature optimum 30 °C, no growth at 45 °C. Optimal growth in saltwater medium but also grows in brackish media containing at least 7 g NaCl and 0.7 g MgCl₂ l⁻¹. Growth rates identical with quinic acid and shikimic acid ($\mu = 0.375 \text{ h}^{-1}$).

DNA base ratio $35.7 \pm 0.1 \text{ mol\% G+C}$. Habitat: marine sediment. Type strain: VenChi2^T (= DSM 6831^T = ATCC BAA-291^T).

Acknowledgements

This study was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG) in the special research program 'Biochemistry of anaerobic bacteria'. The technical assistance of I. Pomper and E. Waltenberger is gratefully acknowledged.

References

- Both, B., Kaim, G., Wolters, J., Schleifer, K. H., Stackebrandt, E. & Ludwig, W. (1991). *Propionigenium modestum*: a separate line of descent within the eubacteria. *FEMS Microbiol Lett* **62**, 53–58.
- Brune, A. & Schink, B. (1992). Anaerobic degradation of hydroaromatic compounds by newly isolated fermenting bacteria. *Arch Microbiol* **158**, 320–327.
- Brune, A., Ludwig, W. & Schink, B. (2002). *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*. *Int J Syst Evol Microbiol* **52**, 441–444.
- Herbert, R. B. (1981). *The Biosynthesis of Secondary Plant Metabolites*. London: Chapman & Hall.
- Janssen, P. H. & Liesack, W. (1995). Succinate decarboxylation by *Propionigenium maris* sp. nov., a new anaerobic bacterium from an estuarine sediment. *Arch Microbiol* **164**, 29–35.
- Lapage, S. P., Sneath, P. H. A., Lessel, E. F., Skerman, V. B. D., Seeliger, H. P. R. & Clark, W. A. (editors) (1992). *International Code of Nomenclature of Bacteria (1990 Revision)*. *Bacteriological Code*. Washington, DC: American Society for Microbiology.
- Ludwig, W. (1995). Sequence databases. In *Molecular Microbial Ecology Manual*, chapter 3.3.5, pp. 1–22. Edited by A. D. L. Akkermans. Dordrecht: Kluwer.
- Ludwig, W. & Strunk, O. (1996). ARB: a software environment for sequence data. <http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/arb.ps>
- Ludwig, W., Kirchhof, G., Klugbauer, N. & 12 other authors (1992). Complete 23S ribosomal RNA sequences of Gram-positive bacteria with a low DNA G+C content. *Syst Appl Microbiol* **15**, 487–501.
- Ludwig, W., Rossello-Mora, R., Aznar, R. & 14 other authors (1995). Comparative analysis of 23S rRNA from *Proteobacteria*. *Syst Appl Microbiol* **18**, 164–188.
- Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K. H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554–568.
- Schink, B. (1984). Fermentation of tartrate enantiomers by anaerobic bacteria, and description of two new species of strict anaerobes, *Ruminococcus pasteurii* and *Ilyobacter tartaricus*. *Arch Microbiol* **139**, 409–414.
- Schink, B. (1992). The genus *Propionigenium*. In *The Prokaryotes*, 2nd edn, pp. 3948–3951. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.
- Schink, B. & Pfennig, N. (1982). *Propionigenium modestum* gen. nov. sp. nov., a new strictly anaerobic, nonsporeforming bacterium growing on succinate. *Arch Microbiol* **133**, 209–216.
- Springer, N., Ludwig, W., Drozanski, W., Amann, R. & Schleifer, K. H. (1992). The phylogenetic status of *Sarcobium lyticum*, an obligate intracellular bacterial parasite of small amoebae. *FEMS Microbiol Lett* **75**, 199–202.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Stieb, M. & Schink, B. (1984). A new 3-hydroxybutyrate fermenting anaerobe, *Ilyobacter polytropus*, gen. nov. sp. nov., possessing various fermentation pathways. *Arch Microbiol* **140**, 139–146.
- Yoshida, S., Tazaki, K. & Minamikawa, T. (1975). Occurrence of shikimic and quinic acids in angiosperms. *Phytochemistry* **14**, 195–197.