

ORIGINAL PAPER

Christof Holliger · Dittmar Hahn · Hermie Harmsen
Wolfgang Ludwig · Wolfram Schumacher
Brian Tindall · Francisco Vazquez · Norbert Weiss
Alexander J. B. Zehnder

***Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration**

Received: 14 July 1997 / Accepted: 27 November 1997

Abstract The highly enriched anaerobic bacterium that couples the reductive dechlorination of tetrachloroethene to growth, previously referred to as PER-K23, was obtained in pure culture and characterized. The bacterium, which does not form spores, is a small, gram-negative rod with one lateral flagellum. It utilized only H₂ as an electron donor and tetrachloroethene and trichloroethene as electron acceptors in an anaerobic respiration process; it could not grow fermentatively. Acetate served as a carbon source in a defined medium containing iron as the sole trace element, the two vitamins thiamine and cyanocobalamin, and the three amino acids arginine, histidine, and threonine. The cells contained menaquinones and *b*-type cytochromes. The G+C content of the DNA was 45.3 ± 0.3 mol%. The cell wall consisted of type-A3 γ peptidoglycan with LL-diaminopimelic acid and one glycine as an

interpeptide bridge. The cells are surrounded by an S-layer; an outer membrane was absent. Comparative sequence analysis of the 16S rRNA sequence showed that PER-K23 is related to gram-positive bacteria with a low G+C content of the DNA. Based on the cytological, physiological, and phylogenetic characterization, it is proposed to affiliate the isolate to a new genus, *Dehalobacter*, with PER-K23 as the type strain of the new species *Dehalobacter restrictus*.

Key words Anaerobic respiration · Reductive dechlorination · Tetrachloroethene · Trichloroethene · Hydrogen oxidation · *Dehalobacter restrictus*

Introduction

Reductive dechlorination of chlorinated compounds such as polychlorinated biphenyls, chlorobenzenes, and chloroethenes has been observed to occur in different anoxic environments. Although there have been many indications that reductive dechlorination reactions are catalyzed by bacteria that couple this reduction to growth (Holliger and Schraa 1994), the isolation of these bacteria appears to be very difficult. Reductive dechlorination as a respiratory process was first shown to occur with the sulfate-reducing bacterium *Desulfomonile tiedjei* (DeWeerd et al. 1990; Dolfig 1990; Mohn and Tiedje 1990, 1991). This bacterium has been isolated from an enrichment with 3-chlorobenzoate as the sole carbon and energy source (Shelton and Tiedje 1984). Besides *Desulfomonile tiedjei*, five other bacteria [*Desulfitobacterium dehalogenans* (Utkin et al. 1994), *Desulfitobacterium chlororespirans* (Sanford et al. 1996), *Desulfitobacterium hafniense* (Christiansen and Ahring 1996), *Desulfitobacterium frappieri* (Bouchard et al. 1996), and strain 2CP-1 (Cole et al. 1994)] have been isolated with chlorinated aromatic compounds as electron acceptors.

Dehalospirillum multivorans (Scholz-Muramatsu et al. 1995), *Dehalococcoides ethenogenes* (Maymo-Gatell et al. 1997), *Desulfitobacterium* sp. strain PCE1 (Gerritse et

C. Holliger (✉) · W. Schumacher · F. Vazquez
Swiss Federal Institute for Environmental Science
and Technology (EAWAG), Limnological Research Center,
CH-6047 Kastanienbaum, Switzerland
Tel. +41-41-349-2145; Fax +41-41-349-2168
e-mail: christof.holliger@eawag.ch

A. J. B. Zehnder
Swiss Federal Institute for Environmental Science
and Technology (EAWAG), Ueberlandstrasse 133,
CH-8600 Dübendorf, Switzerland

D. Hahn
Swiss Federal Institute for Technology (ETH),
Institute for Terrestrial Ecology (ITÖ), Grabenstrasse 3,
CH-8952 Schlieren, Switzerland

H. Harmsen
Department of Microbiology, Agricultural University,
Hesselink van Suchtelenweg 4, NL-6703 CT Wageningen,
The Netherlands

W. Ludwig
Lehrstuhl für Mikrobiologie, Technische Universität München,
D-80290 München, Germany

B. Tindall · N. Weiss
Deutsche Sammlung für Mikroorganismen (DSM),
Mascheroder Weg, D-38124 Braunschweig, Germany

al. 1996), and the three isolates TT4B (Krumholz et al. 1996), MS-1 (Sharma and McCarty 1996), and TEA (Wild et al. 1996) utilize tetrachloroethene or trichloroethene as electron acceptors. Although these organisms share the property of anaerobic respiration with chlorinated ethenes, they are affiliated with different phylogenetic groups and have quite different physiological properties. The use of tetra- and trichloroethene as terminal electron acceptors has also been described for an enrichment culture (Holliger et al. 1993) obtained from an anaerobic, tetrachloroethene-dechlorinating, packed-bed column filled with Rhine River sediment and ground anaerobic granular sludge from a sugar refinery (De Bruin et al. 1992). In this study, the isolation of the tetrachloroethene-dechlorinating bacterium of this enrichment culture, previously referred to as PER-K23, is described. The isolate was characterized with respect to morphological, cytological, and physiological properties, and its phylogenetic position was assigned by comparative sequence analysis of the 16S rRNA.

Materials and methods

Source of inoculum, cultivation procedure, and isolation

PER-K23 was isolated from a previously described tetrachloroethene-dechlorinating enrichment culture (Holliger et al. 1993). For the enrichment culture, material from a packed-bed column that was wet-packed with anaerobic Rhine River sediment and ground anaerobic granular sludge was used as the inoculum (De Bruin et al. 1992). A phosphate-bicarbonate-buffered medium containing the following (in g/l, unless stated otherwise) was used: K_2HPO_4 , 0.65; $NaH_2PO_4 \cdot 2 H_2O$, 0.20; NH_4HCO_3 , 0.44; $CaCl_2 \cdot 2 H_2O$, 0.11; $MgCl_2 \cdot 6 H_2O$, 0.10; $NaHCO_3$, 3.73; $Na_2S \cdot 9 H_2O$, 0.24; resazurin, 0.0005; 1 ml trace element solution (containing in mg/l: $FeCl_2 \cdot 4 H_2O$, 2,000; $MnCl_2 \cdot 4 H_2O$, 100; $CoCl_2 \cdot 6 H_2O$, 190; $ZnCl_2$, 70; $CuCl_2$, 2; $AlCl_3 \cdot 6 H_2O$, 10; H_3BO_3 , 6; Na_2MoO_4 , 36; $NiCl_2 \cdot 6 H_2O$, 24; $Na_2EDTA \cdot 2 H_2O$, 500; and 1 ml concentrated HCl); 1 ml vitamin solution (mg/l medium: biotin, 0.05; folic acid, 0.02; pyridoxine, 0.1; riboflavin, 0.05; thiamine, 0.1; cyanocobalamin, 0.1; nicotinamide, 0.55; *P*-aminobenzoic acid, 0.25; lipoic acid, 0.05; and pantothenic acid, 0.05); $CH_3COONa \cdot 3 H_2O$, 0.68; and peptone, 0.05. The gas phase consisted of H_2 : CO_2 (90:10, v/v) at a pressure of 1.5×10^5 Pa. An aliquot of 20 ml tetrachloroethene dissolved in hexadecane (1 M) per liter of medium was added by syringe; this resulted in a nominal concentration of 20 mM.

In contrast to the medium described above, the defined medium contained only iron ($FeCl_2 \cdot 4 H_2O$, 1.5 mg/l) as a trace element, the two vitamins thiamine and cobalamin (0.1 and 0.5 mg/l medium, respectively), and the three amino acids arginine, histidine, and threonine (8.7, 7.8, and 6 mg/l, respectively). The defined medium was specified by a monofactorial analysis in batch culture experiments by stepwise omitting one trace element, one vitamin, or one amino acid. Omitted supplements were considered not to be essential if growth still occurred after the third or fourth transfer. For specification of the trace element requirement, all glassware was thoroughly treated with 2 M HCl and equilibrated with double-distilled, metal-free water. Where possible, the different stock solutions for the medium were treated with Chelex 100 (BioRad) and diluted with double-distilled, metal-free water.

Single colonies were isolated from agar shakes prepared in tubes filled with 10 ml medium containing 1% agar. The tubes were sealed with viton stoppers, and the gas phase was H_2 : CO_2 (80:20, v/v). Aliquots of 100 μ l of 10^{-5} or 10^{-6} dilutions of the liquid enrichment culture were added to the tubes kept at 42 °C and

containing liquid agar. After mixing, the tubes were laid in a cold water bath in such a way that the agar had a sloped surface after solidification. An aliquot of 100 μ l tetrachloroethene dissolved in hexadecane was added, and the tubes were incubated at 30 °C. Finally, the colonies that appeared were transferred into liquid medium by using sterile Pasteur pipettes.

Electron microscopy

For shadow-casting with palladium, a formvar-coated copper grid (150 mesh, \varnothing 3 mm) was laid on a drop of cell suspension of unfixed cells for 5 min. The air-dried grid was coated with a 1- to 2-nm-thick layer of palladium (amount vaporized, 18 mg) in a Balzers Coater BAE 80T at 5×10^{-5} mbar with a fixed angle of 30° from a distance of 15 cm. The samples were examined with a Philips CM 12 transmission electron microscope.

Thin-sections were prepared as described by Wehrli and Egli (1988). Freeze-etch preparation occurred according to Müller et al. (1980). Replicas of propane-jet-frozen and subsequently freeze-etched samples were used to analyze surface structures.

Extraction and analysis of respiratory lipoquinones

Respiratory lipoquinones were extracted from 100 mg of freeze-dried cells using the two-stage method described by Tindall (1990a,b). The lipoquinones were separated into their different classes (menaquinones and ubiquinones) by TLC on silica gel (Macherey-Nagel no. 805 023) using hexane:*tert*-butylmethylether as the solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were excised from the plate and further analyzed by HPLC (LCD Analytical HPLC; reversed-phase column Macherey-Nagel, 2×125 mm, 3 μ m, RP 18). Methanol served as the eluent, and respiratory lipoquinones were detected at 269 nm.

Phylogenetic analysis

A fragment of approximately 1,400 bp of the 16S rRNA gene was amplified by PCR, cloned into M13mp18/19 (Nölling et al. 1993) and sequenced (Teske et al. 1994). The sequence was deposited under Genbank accession no. U84497. It was added to an alignment of available homologous primary structures from bacteria (Maidak et al. 1994, Van de Peer et al. 1994) using the alignment tool of the ARB program package (W. Ludwig and O. Strunk, <http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/arb.ps>). Phylogenetic analyses were performed by applying distance matrix (ARB, PHYLIP Felsenstein 1982), maximum parsimony (ARB, PHYLIP), and maximum likelihood methods (fastDNAm1; Maidak et al. 1994) on different data sets. Distance matrix and maximum parsimony analyses were performed using data sets comprising all available 16S rRNA primary structures from Gram-positive bacteria with a low DNA G+C content as well as reference sequences from selected representatives of each of the remaining major phylogenetic groups of bacteria. Maximum likelihood methods were used to analyze subsets of approximately 50 sequences comprising the homologous sequences of the nearest neighbors of the organisms studied here and of selected outgroup references from other phylogenetic groups. The data sets varied with respect to the reference sequences and the alignment position included. The variabilities of the individual alignment positions were determined using the respective tool of the ARB package and were used as a criterion to successively remove highly variable positions from the data set. This was done to recognize and minimize treeing artefacts resulting from alignment errors, database inconsistencies, and "false" identities (multiple base changes) in highly variable regions.

Other methods

The Gram type was determined by standard procedures (Doetsch 1981) and by the KOH method (Gregersen 1978). Preparation of

the cell walls and determination of the peptidoglycan type were carried out as described by Schleifer and Kandler (1972). The determination of the G+C content was done as described by Meshbah et al. (1989). Analyses of chloroethenes, chloride, and hydrogen were performed as described previously (Holliger et al. 1993). The presence of cytochromes was tested by recording difference spectra of dithionite-reduced *minus* air-oxidized whole cells and cell fractions. The presence of heme groups in whole cells or membrane fractions (prepared by centrifugation at $200,000 \times g$ for 1 h at 4°C) was analyzed by the pyridine Fe(II) hemochrome technique (Fuhrop and Smith 1975). The bacterial dry weight was determined according to Cypionka and Pfennig (1986) with cells in the early and late exponential growth phases. Superoxide dismutase activity was assayed by superoxide-dismutase-dependent inhibition of horse heart cytochrome *c* reduction (Hyland et al. 1983). Catalase was determined according to Gerhardt et al. (1994) by the observation of pronounced gas evolution after the addition of H_2O_2 to a cell pellet and with *Escherichia coli* as a positive control.

Results

Isolation of PER-K23

As previously described, PER-K23 was enriched from a tetrachloroethene-dechlorinating, packed-bed column filled with Rhine River sediment and ground anaerobic granular sludge from a sugar refinery. A microscopically pure culture was obtained with serial dilutions; the culture grew on H_2/CO_2 only when tetrachloroethene or trichloroethene was present as the electron acceptor. However, when the cultures were transferred to Wilkins-Chalgren anaerobic broth medium, a bacterium that was not able to dechlorinate tetrachloroethene grew (Holliger et al. 1993). First attempts to cultivate PER-K23 on agar-solidified medium to obtain colonies of single cells failed. The determination of the optimal growth temperature indicated that PER-K23 was sensitive to temperatures above 35°C . Therefore, special care was taken to cool down the liquid soft-agar as far as possible (approximately 42°C) and to keep the contact time of the cells with these temperatures as short as possible. With this procedure, single colonies able to dechlorinate tetrachloroethene after transfer to liquid medium were obtained in agar shakes. No growth occurred if these cultures were transferred to anaerobic broth medium. Hence, PER-K23 was then considered to be a pure culture.

Morphology and cytological characterization

PER-K23 was a motile, straight rod with tapered ends (Fig. 1). Cells occurred single or in pairs, were $2\text{--}3\ \mu\text{m}$ long with a diameter of $0.3\text{--}0.5\ \mu\text{m}$, and had one lateral flagellum. In starved cultures, no spores were observed. In some cells, an electron-dense granule was present (Fig. 1). The Gram stain was negative. However, a gram-negative cell-envelope profile was not visible in ultra-thin sections. Rather, the latter indicated the presence of a proteinaceous surface layer (S-layer) (Fig. 2a). This observation was confirmed by electron micrographs of freeze-etch preparations that, in addition, showed a hexagonal

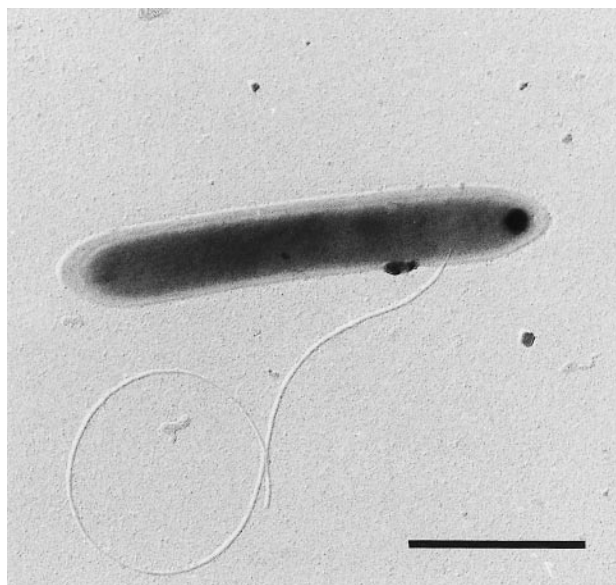


Fig. 1 Electron micrograph of a shadow-casted cell of PER-K23 (bar $1\ \mu\text{m}$)

arrangement of the S-layer protein (Fig. 2b). The cell wall contained the peptidoglycan type A3 γ (Fig. 3) with the amino acids alanine (Ala), D-glutamic acid (Glu), glycine, and LL-diaminopimelic acid (Dpm). Glycine formed an interpeptide bridge between positions 3 and 4 of the oligopeptides L-Ala-D-Glu-LL-Dpm-D-Ala.

Dithionite-reduced *minus* air-oxidized difference spectra of whole cells, cell extracts, or membrane fractions did not indicate the presence of cytochromes. However, the same procedure applied to a membrane extract indicated the presence of *b*-type cytochromes with absorption maxima at 425 and 560 nm (Fig. 4). The pyridine Fe(II) hemochrome extract showed a UV/Vis difference spectrum with absorbance maxima at 557 and 524 nm and a minimum at 540 nm, indicative of the presence of iron protoporphyrin IX, the cofactor of *b*-type cytochromes. TLC showed that menaquinones were the only quinones present. HPLC analysis demonstrated that the major menaquinones were MQ-7 and MQ-8, and that smaller amounts of MQ-6 and MQ-9 were also present. The DNA base composition of PER-K23 was $45.3 \pm 0.3\ \text{mol}\% \text{ G+C}$.

Nutritional requirements, physiology, and growth conditions

Fermented yeast extract, a medium-supplement used for enrichment of PER-K23, could be replaced by peptone in cultures of the isolate. However, growth with peptone as the source of growth factors was only possible if acetate was added as a carbon source. Succinate could not be utilized as carbon source. Growth of the culture depended on the presence of the three amino acids arginine, histidine, and threonine, which could replace the peptone supplement. From the trace elements and vitamins added, only

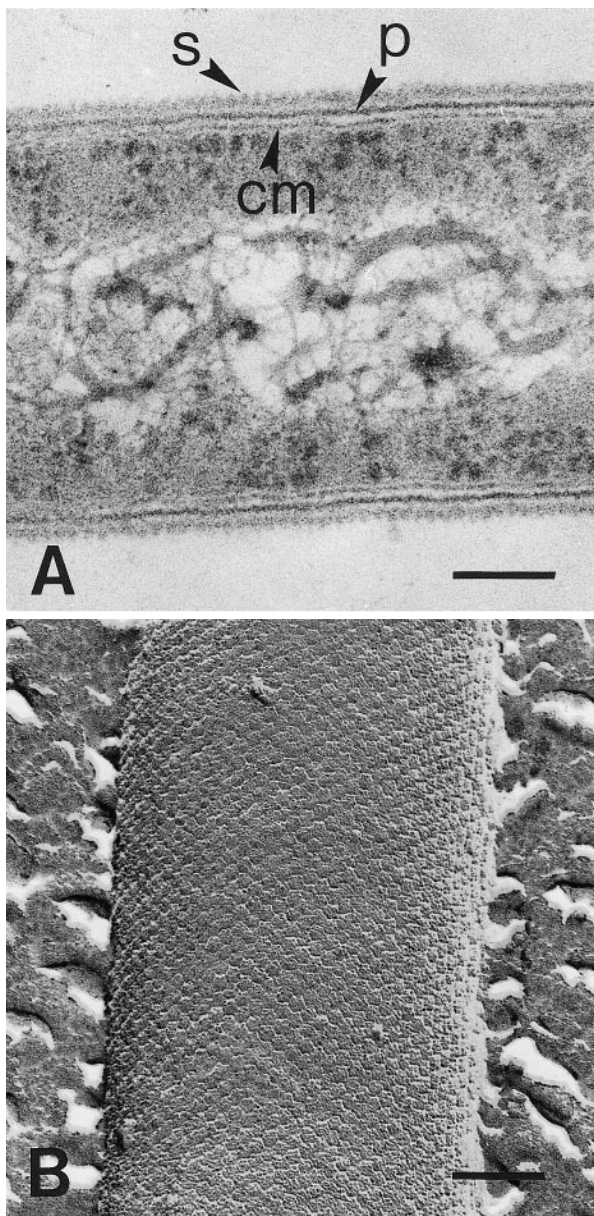


Fig. 2 **A** Electron micrograph of a thin-section of PER-K23 demonstrating a cell wall consisting of a cytoplasmic membrane (*cm*), peptidoglycan (*p*), and a surface layer (*s*) (*bar* 0.1 μm). **B** Electron micrograph of a freeze-etch preparation of PER-K23 (*bar* 0.1 μm)

iron, thiamine, and cyanocobalamin were required for growth of PER-K23.

Among many electron donors tested (H_2 , formate, lactate, pyruvate, propionate, butyrate, acetate, succinate, fumarate, glycine, alanine, aspartate, glutamate, methanol, ethanol, propanol, glucose, fructose, xylose, glycerol, acetoin, and CO), the enrichment culture utilized only dihydrogen and formate for growth with tetrachloroethene as the electron acceptor (Holliger et al. 1993). In contrast to the enrichment, the pure culture of PER-K23 was no longer able to grow with formate as the electron donor. Hence, the only electron donor that supported growth was

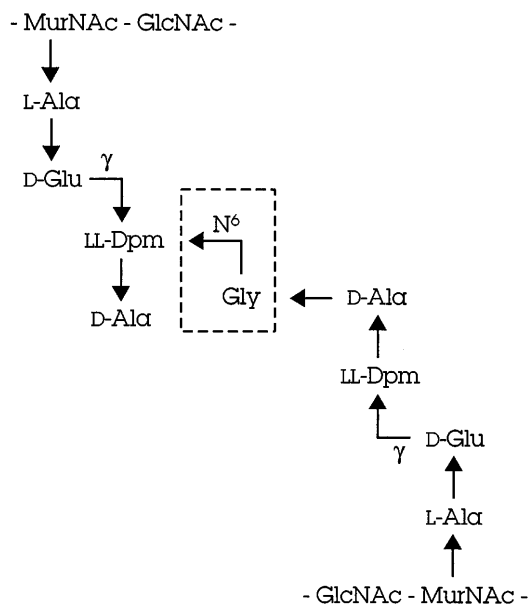


Fig. 3 Fragment of the primary structure of the peptidoglycan of PER-K23. The interpeptide bridge is marked by a *dashed frame*

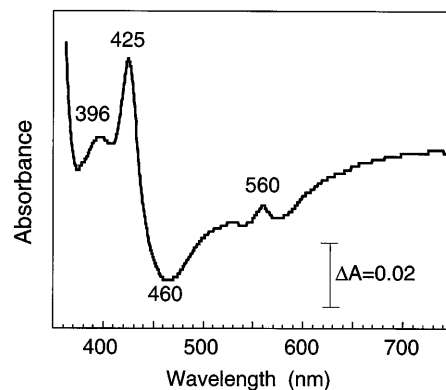


Fig. 4 Redox difference spectrum of membrane extract of PER-K23. Membranes were extracted with octyl- β -D-glucopyranoside, and spectra of dithionite-reduced *minus* air-oxidized extracts were recorded. The protein concentration was 1.36 mg/ml

dihydrogen. Fermentative growth was also not observed. Negative test results with nitrate, sulfate, and other compounds in the enrichment culture had previously indicated that PER-K23 cannot use many electron acceptors (Holliger et al. 1993). Experiments with Fe(III) , Mn(IV) , and N_2O in the presence of molecular hydrogen confirmed the previous result that PER-K23 utilizes only tetra- and trichloroethene as electron acceptors. PER-K23 neither contained superoxide dismutase nor exhibited catalase activity.

Under optimal growth conditions (30°C ; initial pH, 7.2) in gently shaken cultures with medium containing acetate as the carbon source and peptone, PER-K23 grew with a specific growth rate of $0.037 \pm 0.01 \text{ h}^{-1}$ ($t_d = 19 \text{ h}$). The growth yield determined by measuring the production of cellular dry weight was $3.3 \pm 0.3 \text{ g dry wt. per mol of Cl}^-$ formed.

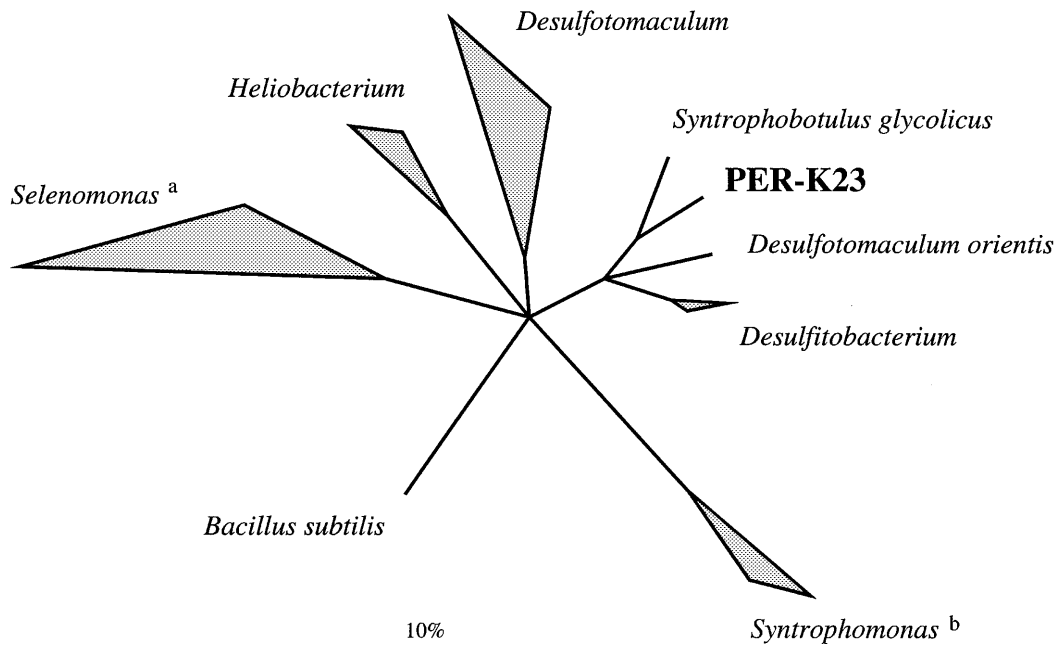


Fig. 5 16S rRNA-based phylogenetic tree reflecting the relationships of PER-K23. The tree was reconstructed applying a maximum likelihood approach including sequence positions that contain identical residues in at least 50% of available 16S rRNA sequences from the gram-positive bacteria with a low DNA G+C content. The topology was corrected according to the results of the distance matrix and maximum parsimony analyses. Given that only partial sequence data are available in the case of *Desulfotomaculum orientis*, its position in the tree has to be regarded as preliminary. The triangles indicate groups of phylogenetically related species or genera (bar 10% estimated sequence divergence). The *Selenomonas* group (a) comprises *Acidaminococcus*, *Dialister*, *Megasphaera*, *Mitsuokella*, *Phascolarctobacterium*, *Selenomonas*, *Sporomusa*, *Succiniclasticum*, *Veillonella*, and *Zymophilus*. The *Syntrophomonas* group (b) comprises *Syntrophomonas* and *Syntrophospora*.

Most analyses supported a moderate relationship of this group to *Heliobacterium* (Woese et al. 1985), the remaining desulfotomaculi (Delaney et al. 1989; Patel et al. 1992; Redburn and Patel 1993), the *Syntrophospora-Syntrophomonas* branch (Zhao et al. 1990), and other “phylogenetically gram-positive” bacteria with gram-negative cell walls such as *Acidaminococcus*, *Dialister*, *Megasphaera*, *Pectinatus*, *Phascolarctobacterium*, *Selenomonas*, *Veillonella*, and *Zymophilus* (Schleifer et al. 1990; Zhao et al. 1990; Del Dot et al. 1993; Cook et al. 1994; Willems et al. 1995;). However, the significance of such a grouping is low, as indicated by the multifurcation in Fig. 5.

Phylogeny

The phylogenetic relationship of PER-K23 to other bacteria is depicted in Fig. 5. Not included in this analysis is the isolate TEA, for which a very high sequence similarity of 99.7% has been shown previously (Wild et al. 1996). Within the phylogenetic group of the gram-positive bacteria with a low DNA G+C content, *Syntrophobotulus glycolicus*, with an overall 16S rRNA sequence similarity of 92.9%, is the closest relative of PER-K23. PER-K23, isolate TEA, and *S. glycolicus* together with five desulfitobacteria (Utkin et al. 1994; Bouchard et al. 1996; Christiansen and Ahring 1996; Gerritse et al. 1996; Sanford et al. 1996) and *Desulfotomaculum orientis* (Delaney et al. 1989) are members of a phylogenetic cluster defined by sequence similarities of 88.5% and higher. The corresponding values for these organisms and other members of the major phylogenetic group of the gram-positive bacteria with a low DNA G+C content are slightly lower (82.2–87.5%).

Discussion

PER-K23 had almost the same physiological properties have been as described previously for the dominating organism in the enrichment culture (Holliger et al. 1993). Properties of both pure and enrichment culture differed only in the spectrum of electron donors utilized. In contrast to the enrichment culture, PER-K23 was no longer able to grow with formate as an electron donor. The only electron donor that supported growth was molecular hydrogen. This result suggests that growth on formate in the enrichment culture was probably due to the presence of a contaminating bacterium that had formate lyase activity, producing dihydrogen and carbon dioxide. Similar narrow ranges of electron donors utilized are known only for some methanogens (Vogels et al. 1988) and for two tetrachloroethene-dechlorinating bacteria, *Dehalococcoides ethenogenes* (Maymo-Gatell et al. 1997) and isolate TEA (Wild et al. 1996). 16S rRNA sequence analysis, however, clearly showed that PER-K23, strain TEA, and *Dehalococcoides ethanogenes* belong to the domain Bacteria, and not to the Archaea.

Table 1 Comparison of tetrachloroethene-dechlorinating bacteria (*nr* not reported)

Property	<i>Dehalobacter restrictus</i> strain PER-K23	<i>Desulfito-bacterium</i> sp. strain PCE1 ^a	<i>Dehalo-spirillum multivorans</i> ^b	<i>Dehalo-coccoides ethenogenes</i> strain 195 ^c	Isolate TEA ^d	Isolate TT4B ^e	Isolate MS-1 ^f
Morphology	Rod	Curved rod	Spirillum	Irregular coccus	Rod	Rod	Rod
Motility	Motile, 1 lateral flagellum	Motile, 4 lateral flagella	Motile and nonmotile strains	nr	Motile, 1–4 lateral flagella	Nonmotile	Motile, peritrichous fimbriae
Gram staining	Negative	Positive	Negative	nr	Negative	Negative	Negative
S-layer	Yes	Yes	No	Yes	Yes	nr	nr
Spores	No	No	No	nr	No	No	No
Cytochromes	Type <i>b</i>	Type <i>c</i>	Types <i>b</i> and <i>c</i>	nr	nr	nr	nr
Quinones	Menaquinones	nr	Menaquinones	nr	nr	nr	nr
G+C content	45.3	nr	41.5	nr	nr	nr	nr
Temperature optimum	25–30 °C	34–38 °C	30 °C	nr	nr	21–31 °C	37 °C
pH optimum	6.8–7.6	7.2–7.8	7.0–7.5	nr	nr	7.4	7.0
<i>t</i> _d ^g	19 h	58 h	2.5 h	19 h	nr	48–96 h	nr
Yield (g protein/mol Cl ⁻)	2.1	1.6 ^h	1.4	4.8	0.1–0.25	0.15	nr
Phylogeny	Gram-positive bacteria with low G+C content	Gram-positive bacteria with low G+C content	ε-Subdivision of Proteobacteria	Yet unclear	Gram-positive bacteria with low G+C content	δ-Subdivision of Proteobacteria	Enterobacteriaceae
Electron donors	H ₂ ⁱ	Formate, lactate, pyruvate, ethanol, butyrate, succinate	H ₂ ⁱ , formate ⁱ , lactate, H ₂ ⁱ , pyruvate, ethanol, glycerol	H ₂ ^j	H ₂ ⁱ	Acetate, pyruvate	Polymers, carbohydrates, esters, carboxylic acids, amides, amino acids, aromatics, alcohols ^k
Electron acceptors	PCE, TCE ^l	PCE, ortho-chlorinated phenolic compounds, fumarate, sulfite, thiosulfate	PCE, TCE, fumarate, nitrate ^m	PCE, TCE	PCE, TCE	PCE, TCE, fumarate, Fe(III)NTA	O ₂ , nitrate
Fermentative growth	No	Yes ⁿ	Yes ⁿ	No	No	No	Yes
Respiratory growth with PCE ^o	Yes	Yes	Yes	Yes	Yes	Yes	Uncertain ^p
Product of PCE dechlorination	<i>cis</i> -1,2-DCE	TCE ^q	<i>cis</i> -1,2-DCE	Ethene	<i>cis</i> -1,2-DCE	<i>cis</i> -1,2-DCE	<i>cis</i> -1,2-DCE
Nutritional requirements ^f	Arginine, histidine, threonine ^s	None	None	Yes ^t	None	None	None
Isolated from	Rhine River sediment mixed with granular sludge	Soil polluted with chlorinated ethenes	Activated sludge	Digested sludge	Contaminated aquifer	Stream sediment with TCE	Aquifer material from a PCE-contaminated site

^aGerritse et al. (1996)^bScholz-Muramatsu et al. (1995)^cMaymo-Gatell et al. (1997)^dWild (1995) and Wild et al. (1996)^eKrumholz et al. (1996)^fSharma and McCarty (1996)^gDuring growth with PCE^hWith 3-chloro-4-hydroxyphenylacetate as electron acceptorⁱOnly with acetate as carbon source^jAcetate present, but not known whether needed as carbon source^kFor a more detailed list, see Sharma and McCarty (1996)^lPCE = tetrachloroethene, TCE = trichloroethene^mYeast-extract-dependentⁿWith pyruvate as substrate^oPCE-dependent growth with a nonfermentable substrate^pPCE-dependent growth not yet demonstrated^qOnly small amounts of *cis*- and *trans*-1,2-DCE (< 5% of PCE consumed)^rExcept vitamins^sOnly thiamine and cobalamin are needed as vitamins^tNot yet identified

The 16S rRNA data have already shown previously that PER-K23 and isolate TEA have very high sequence similarities (Wild et al. 1996). This, together with the almost identical physiology, the same morphology, and the presence of an S-layer, indicates that these bacteria belong to the same genus, perhaps even to the same species. There were, however, some differences between the isolates. The molar growth yield of isolate TEA was approximately 10- to 20-fold lower, an observation that cannot be easily explained. PER-K23, on the other hand, required three amino acids as additional growth factors, a property that has not been reported for isolate TEA.

The 16S rRNA analysis also revealed that PER-K23 is closely related to *S. glycolicus*, which has been assigned to the gram-positive bacteria with a low DNA G+C content (Friedrich et al. 1996). The physiological properties of PER-K23 and *S. glycolicus*, however, are quite different. PER-K23 cannot ferment glyoxylate and glycolate, and cannot utilize glyoxylate as electron acceptor, whereas *S. glycolicus* cannot grow with tetrachloroethene as an electron acceptor and dihydrogen as an electron donor. In the presence of dihydrogen, *S. glycolicus* is able to reduce glyoxylate and to produce ATP, but it does not grow upon this reaction (Friedrich and Schink 1995).

To date, seven pure cultures that dechlorinate tetrachloroethene at high rates and utilize the chlorinated compound as an electron acceptor have been obtained. Apart from this common feature, they seem to be quite different from each other and from the PER-K23 described in this study (Table 1). The tetrachloroethene-dechlorinating bacteria isolated so far belong to five phylogenetically different groups of bacteria. Physiologically they range from facultative anaerobes, nitrate reducers, and sulfoxy anion reducers to strict tetrachloroethene reducers. They were isolated from quite different environments, e.g., activated sludge, sediments, and aquifers. Strain MS-1, a facultative anaerobe for which tetrachloroethene-dependent growth has not yet been demonstrated, was able to oxidize a broad spectrum of substrates. PER-K23, isolate TEA, and *Dehalococcoides ethenogenes*, on the other hand, could only utilize dihydrogen, and the other three tetrachloroethene dechlorinators could use two to six different electron donors.

Three isolates (*Desulfitobacterium* sp. strain PCE1, isolate TEA, and PER-K23) belong phylogenetically to the gram-positive bacteria with a low DNA G+C content. All have lateral flagella, but *Desulfitobacterium* sp. strain PCE1 has a different morphology. The Gram staining was positive for strain PCE1, and negative for isolate TEA and PER-K23. A common feature of the cell envelope of all three bacteria was the presence of an S-layer. The peptidoglycan of *Desulfitobacterium* sp. strain PCE1 and PER-K23 contains LL-diaminopimelic acid, an unusual feature of gram-positive bacteria. It is not known whether the peptidoglycan of *Desulfitobacterium* sp. strain PCE1 also consists of the A3 γ type, as it was found for PER-K23. Within the gram-positive bacteria, only some strains of *Propionibacterium* (a genus of bacteria not closely related to *Desulfitobacterium* sp. strain PCE1 and PER-K23) are

known to have the A3 γ -type peptidoglycan (Schleifer and Kandler 1972). In contrast to *Desulfitobacterium* sp. strain PCE1, which contained *c*-type cytochromes, PER-K23 had *b*-type cytochromes. Strain PCE1 dechlorinated tetrachloroethene mainly to trichloroethene and is physiologically much more versatile than isolate TEA and PER-K23. On the basis of electron donors and electron acceptors utilized, strain PCE1 had much more in common with *Dehalospirillum multivorans*, a member of the ϵ -subdivision of Proteobacteria. With respect to these two organisms, differences in the inorganic electron acceptors used for growth are the physiologically most distinctive features.

Dehalospirillum multivorans and PER-K23 showed similarities regarding the tetrachloroethene dechlorination. Both organisms dechlorinated tetrachloroethene to *cis*-1,2-dichloroethene and could couple this reaction to dihydrogen oxidation, and both contained *b*-type cytochromes and menaquinones that are possibly involved in electron transfer (Schumacher and Holliger 1996). In both organisms, tetrachloroethene dechlorination was photoreversibly inhibited by 1-iodopropane, indicating that a corrinoid was involved in the dechlorination (Neumann et al. 1995; Schumacher and Holliger 1996). The purified tetrachloroethene reductive dehalogenase of *Dehalospirillum multivorans* and the tetrachloroethene reductase of PER-K23 were indeed corrinoid-containing enzymes (Neumann et al. 1996; Schumacher et al. 1997). A major difference between the two organisms has been found in the localization of the tetrachloroethene-reducing enzyme. While the tetrachloroethene reductive dehalogenase of *Dehalospirillum multivorans* is localized in the cytoplasmic fraction (Neumann et al. 1994), the tetrachloroethene reductase of PER-K23 is membrane-bound (Schumacher and Holliger 1996).

Due to the distinctive morphological, cytological, and physiological properties of PER-K23 and the comparative sequence analysis of its 16S rRNA, we propose to assign PER-K23 to a new genus and species, *Dehalobacter restrictus*.

Description of *Dehalobacter* gen. nov.

Dehalobacter (gen. nov.) De.ha.lo.bac'ter. L.pref. *de*, from; Gr.n. *halo*, *halos*, the sea, salt; M.L.masc.n. *bacter*, equivalent of Gr.neut.n. *bacterion*, a rod; M.L.masc.n. *Dehalobacter*, a halogen-removing, rod-shaped bacterium. The description of this genus is the same as that of *Dehalobacter restrictus*, the type species.

Description of *Dehalobacter restrictus* sp. nov.

Dehalobacter restrictus (sp. nov.) re.stric'tus. L.adj. *restrictus*, limited, confined (referring to the limited substrate range utilized). Rod-shaped, motile cells with tapered ends, 0.3–0.5 \times 2–3 μ m. Cells appear singly or in pairs. No spores formed. Cells stain gram-negative. One

lateral flagellum per cell. Cells are surrounded by an S-layer. Cell wall peptidoglycans contain L-alanine, D-glutamic acid, LL-diaminopimelic acid, and glycine and are cross-linked between positions 3 and 4 with a glycine interpeptide bridge (type A3 γ). Menaquinones and b-type cytochromes are present. The G+C content of the DNA is 45.3 ± 0.3 mol%.

Strict anaerobe. No catalase, no superoxide dismutase. Chemolithoheterotroph. Only H₂ is used as electron donor with tetra- or trichloroethene as terminal electron acceptors that are dechlorinated to *cis*-1,2-dichloroethene. Lactate, pyruvate, propionate, butyrate, acetate, formate, succinate, fumarate, glycine, alanine, aspartate, glutamate, methanol, ethanol, propanol, glucose, fructose, xylose, glycerol, acetoin, and CO are not utilized in the presence and in the absence of tetrachloroethene. Nitrate, nitrite, fumarate, dimethyl sulfoxide, trimethylamine N-oxide, Fe(III), Mn(IV), sulfate, sulfite, thiosulfate, and sulfur are not reduced. Acetate serves as carbon source. Growth in a defined medium depends on the presence of iron, thiamine, cyanocobalamin, arginine, histidine, and threonine. Growth was optimal between 25 and 35 °C. No growth at 10 °C and below, or at 37 °C and above. Same growth rates at pH values between 6.8–7.6; no growth at pH 6.5 and below, or at pH 8.0 and above.

The type strain *Dehalobacter restrictus* strain PER-K23 was isolated from a tetrachloroethene-dechlorinating, packed-bed column that was filled with Rhine River sediment and ground anaerobic granular sludge from a sugar refinery. The organism was enriched on H₂/CO₂ and tetrachloroethene dissolved in hexadecane.

The strain has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSM 9455^T).

Acknowledgements The work of W. Schumacher was financially supported by a fellowship from the Swiss National Foundation (grant no. 3100-040855.94/1). We thank A. Taverne-Thiele, J. Groenewegen, and R. Hermann for the electron micrographs, K.-D. Jahnke for the G+C content determination, and B. Schink for valuable discussions.

References

- Bouchard B, Beaudet R, Villemur R, McSween G, Lépine F, Bissillon J-G (1996) Isolation and characterization of *Desulfotobacterium frappieri* sp. nov., an anaerobic bacterium which reductively dechlorinates pentachlorophenol to 3-chlorophenol. *Int J Syst Bacteriol* 46:1010–1015
- Christiansen N, Ahring BK (1996) *Desulfotobacterium hafniense* sp. nov., an anaerobic, reductively dechlorinating bacterium. *Int J Syst Bacteriol* 46:442–448
- Cole JR, Cascarelli AL, Mohn W, Tiedje JM (1994) Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol. *Appl Environ Microbiol* 60:3536–3542
- Cook GM, Rainey FA, Chen G, Stackebrandt E, Russell JB (1994) Emendation of the description of *Acidaminococcus fermentans*, a trans-aconitate- and citrate-oxidizing bacterium. *Int J Syst Bacteriol* 44:576–578
- Cypionka H, Pfennig N (1986) Growth yields of *Desulfotomaculum orientis* with hydrogen in chemostat culture. *Arch Microbiol* 143:396–399
- De Bruin WP, Kotterman MJJ, Posthumus MA, Schraa G, Zehnder AJB (1992) Complete biological reductive transformation of tetrachloroethene to ethane. *Appl Environ Microbiol* 58:1996–2000
- Del Dot T, Osawa R, Stackebrandt E (1993) *Phascolarctobacterium faecium*, gen. nov., spec. nov., a novel taxon of the *Sporomusa* group of *Bacteria*. *Syst Appl Microbiol* 16:380–384
- Delaney M, Widdel F, Stahl DA (1989) Natural relationships among sulfate-reducing eubacteria. *J Bacteriol* 171:6689–6695
- DeWeerd KA, Mandelco L, Tanner RS, Woese CR, Suflita JM (1990) *Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic, dehalogenating, sulfate-reducing bacterium. *Arch Microbiol* 154:23–30
- Doetsch RN (1981) Determinative methods of light microscopy. In: Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (eds) *Manual of methods for general microbiology*. American Society for Microbiology, Washington DC, pp 21–33
- Dolfing J (1990) Reductive dechlorination of 3-chlorobenzoate is coupled to ATP production and growth in anaerobic bacterium, strain DCB-1. *Arch Microbiol* 153:264–266
- Felsenstein J (1982) Numerical methods for inferring evolutionary trees. *Q Rev Biol* 57:379–404
- Friedrich M, Schink B (1995) Electron transport phosphorylation driven by glyoxylate respiration with hydrogen as electron donor in membrane vesicles of a glyoxylate-fermenting bacterium. *Arch Microbiol* 163:268–275
- Friedrich M, Springer N, Ludwig W, Schink B (1996) Phylogenetic positions of *Desulfofustis glycolicus* gen. nov., sp. nov., and *Syntrophobotulus glycolicus* gen. nov., sp. nov., two new strict anaerobes growing with glycolic acid. *Int J Syst Bacteriol* 46:1065–1069
- Fuhrhop JH, Smith KM (1975) Preparation of metalloporphyrins from porphyrins and isolation from natural sources. In: Smith KM (ed) *Porphyrins and metalloporphyrins*, Elsevier, Amsterdam, pp 757–861
- Gerhardt P, Murray RGE, Wood WA, Krieg NR (1994) *Methods for general and molecular bacteriology*. American Society for Microbiology, Washington, DC
- Gerritse J, Renard V, Pedro Gomes TM, Lawson PA, Collins MD, Gottschal JC (1996) *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or *ortho*-chlorinated phenols. *Arch Microbiol* 165:132–140
- Gregersen T (1978) Rapid method for distinction of gram-negative from gram-positive bacteria. *Eur J Appl Microbiol* 5:123–127
- Holliger C, Schraa G (1994) Physiological meaning and potential for application of reductive dechlorination by anaerobic bacteria. *FEMS Microbiol Rev* 15:297–305
- Holliger C, Schraa G, Stams AJM, Zehnder AJB (1993) A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl Environ Microbiol* 59:2991–2997
- Hyland K, Voisin E, Banoun H, Auclair C (1983) Superoxide dismutase assay using alkaline dimethyl sulfoxide as superoxide anion-generating system. *Anal Biochem* 135:280–287
- Krumholz LR, Sharp R, Fishbain SS (1996) A freshwater anaerobe coupling acetate oxidation to tetrachloroethylene dehalogenation. *Appl Environ Microbiol* 62:4108–4113
- Maidak BL et al. (1994) The ribosomal database project. *Nucleic Acids Res* 22:3485–3487
- Maymo-Gatell X, Chien Y, Gossett JM, Zinder SH (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* 276:1568–1571
- Meshbah M, Premachandran U, Whitman W (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *Int J Syst Bacteriol* 39:159–167
- Mohn WW, Tiedje JM (1990) Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation. *Arch Microbiol* 153:267–271

- Mohn WW, Tiedje JM (1991) Evidence for chemiosmotic coupling of reductive dechlorination and ATP synthesis in *Desulfomonile tiedjei*. Arch Microbiol 157:1–6
- Müller M, Meister N, Moor H (1980) Freezing in a propane jet and its application in freeze-fracturing. Mikroskopie 36:129–140
- Neumann A, Scholz-Muramatsu H, Diekert G (1994) Tetrachloroethene metabolism of *Dehalospirillum multivorans*. Arch Microbiol 162:295–301
- Neumann A, Wohlfarth G, Diekert G (1995) Properties of tetrachloroethene and trichloroethene dehalogenase of *Dehalospirillum multivorans*. Arch Microbiol 163:276–281
- Neumann A, Wohlfarth G, Diekert G (1996) Purification and characterization of tetrachloroethene reductive dehalogenase from *Dehalospirillum multivorans*. J Biol Chem 271:16515–16519
- Nölling J, Hahn D, Ludwig W, De Vos WM (1993) Phylogenetic analysis of thermophilic *Methanobacterium* sp.: evidence for a common formate-utilizing ancestor. Syst Appl Microbiol 16:208–215
- Patel BK, Love CA, Stackebrandt E (1992) Helix 6 of the 16S rRNA of the bacterium *Desulfotomaculum australicum* exhibits an unusual structural idiosyncrasy. Nucleic Acids Res 20:5483–5483
- Redburn AC, Patel BK (1993) Phylogenetic analysis of *Desulfotomaculum thermobenzoicum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. FEMS Microbiol Lett 113:81–86
- Sanford RA, Cole JR, Löffler FE, Tiedje JM (1996) Characterization of *Desulfitobacterium chlororespirans* sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. Appl Environ Microbiol 62:3800–3808
- Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36:407–477
- Schleifer KH, Leuteritz M, Weiss N, Ludwig W, Kirchhof G, Seidel RH (1990) Taxonomic study of anaerobic, gram-negative, rod-shaped bacteria from breweries: emended description of *Pectinatus cerevisiiphilus* and description of *Pectinatus frisingsensis* sp. nov., *Selenomonas lactificex* sp. nov., *Zymophilus raffinosivorans* gen. nov., sp. nov., and *Zymophilus paucivorans* sp. nov. Int J Syst Bacteriol 40:19–27
- Scholz-Muramatsu H, Neumann A, Messmer M, Moore E, Diekert G (1995) Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Arch Microbiol 163:48–56
- Schumacher W, Holliger C (1996) The proton/electron ratio of the menaquinone-dependent electron transport from dihydrogen to tetrachloroethene in "*Dehalobacter restrictus*." J Bacteriol 178:2328–2333
- Schumacher W, Holliger C, Zehnder AJB, Hagen WR (1997) Redox chemistry of cobalamin and iron-sulfur cofactors in the tetrachloroethene reductase of *Dehalobacter restrictus*. FEBS Lett 409:421–425
- Sharma PK, McCarty PL (1996) Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenates tetrachloroethene to *cis*-1,2-dichloroethene. Appl Environ Microbiol 62:761–765
- Shelton DR, Tiedje JM (1984) Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Appl Environ Microbiol 48:840–848
- Teske A, Alm E, Regan JM, Toze S, Rittmann BE, Stahl DA (1994) Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J Bacteriol 176:6623–6630
- Tindall BJ (1990a) A comparative study of the lipid composition of *Halobacterium saccharovororum* from various sources. Syst Appl Microbiol 13:128–130
- Tindall BJ (1990b) Lipid composition of *Halobacterium lacusprofundi*. FEMS Microbiol Lett 66:199–202
- Utkin I, Woese C, Wiegel J (1994) Isolation and characterization of *Desulfitobacterium dehalogenans* gen. nov., sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. Int J Syst Bacteriol 44:612–619
- Van de Peer Y, Van den Broeck I, De Rijk P, De Wachter R (1994) Database on the structure of small ribosomal subunit RNA. Nucleic Acids Res 22:3488–3494
- Vogels GD, Keltjens JT, Van der Drift C (1988) Biochemistry of methane production. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. Wiley, New York, pp 707–770
- Wehrli E, Egli T (1988) Morphology of nitrilotriacetate-utilizing bacteria. Syst Appl Microbiol 10:306–312
- Wild A (1995) Anaerobe mikrobielle Dehalogenierung chlorierter Ethene. PhD Thesis, ETH Zurich
- Wild A, Hermann R, Leisinger T (1996) Isolation of an anaerobic bacterium which reductively dechlorinates tetrachloroethene and trichloroethene. Biodegradation 7:507–511
- Willems AMCG, Willems A, Collins MD (1995) Phylogenetic placement of *Dialister pneumosintes* (formerly *Bacteroides pneumosintes*) within the *Sporomusa* sub-branch of the *Clostridium* subphylum of the gram-positive bacteria. Int J Syst Bacteriol 45:403–405
- Woese CR, Debrunner-Vossbrinck BA, Oyaizu H, Stackebrandt E, Ludwig W (1985) Gram-positive bacteria: possible photosynthetic ancestry. Science 229:762–765
- Zhao HX, Yang DC, Woese CR, Bryant MP (1990) Assignment of *Clostridium bryantii* to *Syntrophospora bryantii* gen. nov., comb. nov. on the basis of a 16S rRNA sequence analysis of its crotonate-grown pure culture. Int J Syst Bacteriol 40:40–44