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Detection of the anaerobic dechlorinating microorganism *Desulfomonile tiedjei* in environmental matrices by its signature lipopolysaccharide branched-long-chain hydroxy fatty acids

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Abstract: Desulfomonile tiedjei is a Gram-negative sulfate-reducing bacterium capable of catalyzing aryl reductive dehalogenation reactions. Since many toxic and persistent contaminants in the subsurface are halogenated aromatic compounds, the detection and enumeration of dehalogenating microorganisms in the environment may be a useful tool for planning and evaluating bioremediation efforts. In this study, we show that *D. tiedjei* contains unique lipopolysaccharide branched 3-hydroxy fatty acids, unknown as yet in other bacteria, and that it is possible to detect the bacterium in inoculated aquifer sediments based on these signature lipid biomarkers. The detection of *D. tiedjei* and other dehalogenating microorganisms possessing similar cellular properties in environmental matrices may be possible by this technique. Additionally, the effect of such inoculation on dehalogenation activity is examined.

Key words: Anaerobic dehalogenation; *Desulfomonile tiedjei*; Sulfate-reducing bacterium; Detection in environment; Signature lipid biomarker

Introduction

Aryl reductive dehalogenation is often the initial and rate limiting step in the anaerobic biodegradation of haloaromatic pollutants [1]. Consequently, the detection of dehalogenating microorganisms is critical in determining the dehalogenating potential of various environments and the factors that may best potentiate the growth of the requisite bacteria. Detection of signature lipid biomarkers is a convenient and powerful means by which the presence of small numbers of specific microorganisms in complex environmental matrices can be detected [2]. With

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the isolation of *Desulfomonile tiedjei* [3] and subsequent development of a defined medium for its growth [4], it has become possible to examine a known dehalogenating microorganism for possible signature biomarkers. We report that *D. tiedjei* contains characteristic branched, long-chain hydroxy fatty acids in its lipopolysaccharide that can be used as signature biomarkers. Lipopolysaccharide hydroxy fatty acids (LPS-OHFA) can be readily assessed in environmental matrices by gas chromatographic/mass spectrometric analysis of derivatives after hydrolysis and re-extraction of the lipid residue [5]. Analysis of environments containing haloaromatic contaminants for LPS-OHFA patterns like those of *D. tiedjei* could indicate the presence of related microorganisms with potential aryl reductive dehalogenating activity. A similar analysis could also indicate which conditions best potentiate the growth of *D. tiedjei* in reactors or remediation efforts.

Materials and Methods

Growth of the organism

D. tiedjei was grown in a defined medium without yeast extract as described previously [4]. The fatty acid profiles of the organism were obtained from cells grown with either 40 mM pyruvate, formate, or isovanillate as electron donors and 2 mM 3-chlorobenzoate (3 CBz) or sulfate as electron acceptors at 37°C. Cells were harvested in early stationary phase by centrifugation (15 000 $\times g$ for 20 min at 4°C). Cell pellets were frozen and lyophilized prior to lipid extraction. For the amendment of aquifer sediments, *D. tiedjei* was grown with 40 mM pyruvate and 2 mM 3CBz as electron donor and acceptor, respectively. The cells were harvested anaerobically, and the pellet was washed once in anoxic phosphate buffer (100 mM, pH 7.5). The cell concentration of the resuspension was estimated based on the reported dry weight of *D. tiedjei* cells [6].

Aquifer incubations

Sediment and groundwater were sampled from the methanogenic portion of an anoxic aquifer as previously described [7]. Aquifer incubation mixtures were constructed in an anaerobic glovebox by the addition of 50.0 + 0.5 g sediment and 25 ml of ground water to sterile 160 ml serum bottles. The serum bottles were then closed with a rubber stopper, crimp sealed and placed under a headspace of N_2/CO_2 (80:20; 35 kPa). Total bacterial numbers in these aquifer incubations were estimated as 2×10^9 cells based upon previous enumeration by direct microscopy [7]. Aquifer incubations were inoculated with 2×10^7, 2×10^8 and 2×10^9 of *D. tiedjei* cells. Eight replicates were prepared for each inoculation level. Five replicates of each inoculum level were frozen, lyophilized, randomly coded, and sent as blind samples to the Center for Environmental Biotechnology for subsequent lipid analysis. The remaining three replicates, along with triplicate sterile aquifer incubations (autoclaved for 20 min on consecutive days), were amended with 3 CBz (500 μM), incubated at 25°C, and analyzed periodically for 3 CBz disappearance by HPLC as previously described [8].

Lipid analysis

Lyophilized bacterial cells and aquifer incubation mixtures were extracted with the single-phase chloroform-methanol buffer Bligh and Dyer technique [9] modified to include a phosphate buffer [10]. All samples were extracted at room temperature with 142.5 ml chloroform:methanol:phosphate buffer (50 mM PO_4, pH 7.4) (1:2:0.8, v:v:v) for 3 h. The aquifer samples were extracted in 250 ml glass centrifuge bottles, centrifuged at 650 $\times g$ for 30 min, and the liquid phase was decanted into a separatory funnel. With sandy sediments and sufficient one-phase extractant volume, it is not necessary to wash the sediment for quantitative recovery. For bacterial samples, approximately 20–25 mg dry weight of bacterial cells were added directly to the separatory funnel. Care was taken to prevent artifacts and contamination [11]. To separate the aqueous (upper) and organic (lower) phases, 37.5 ml each of chloroform and distilled water were added, and the two phases were allowed to separate overnight. The organic phase (containing the bacterial lipids) was collected after filtration through a pre-extracted fluted Whatman 2V filter and the

solvent removed by rotary evaporation at 37°C. The total lipid extract was fractionated on silicic acid columns into neutral, glyco- and polar lipids [11]. The polar lipid fraction, recovered in methanol, was transesterified with mild alkaline methanolysis [10], and the methyl esters were then separated, quantified, and tentatively identified by capillary gas chromatography (GC). A Restek RT_x-1 (nonpolar methyl silicone) capillary column (60 m × 0.25 mm i.d. × 0.1 mm film thickness) was used to separate the fatty acid esters on a Shimadzu GC9A equipped with a FID. The compounds were introduced using a 1 μl splitless injection (100 ml min^{-1} after 0.5 min) with a column flow rate of 1 ml min^{-1} using hydrogen as the carrier gas. The column temperature was programed with an initial temperature of 100°C and increased 10°C min^{-1} to 150°C, held 1 min and increased to 282°C at 3°C min^{-} and maintained at this temperature for 5 min. The injector and detector temperatures were set at 290°C. The phospholipid ester-linked fatty acid (PLFA) structures were verified using GC/mass spectrometry on a Hewlett Packard 5890 Series II GC equipped with a HP5971 mass selective detector (MS) operated in the positive ion electron impact mode at an electron energy of 70 eV. The same GC column and program described above were used except the injector temperature was 270°C. Mono and dienoic PLFA double bond positions were determined by GC/MS analysis of the dimethyl disulfide adducts [12].

The LPS-OHFA from the Lipid A were recovered from the extracted lipid residue after hydrolysis in 1 N HCl for 2–4 h at 100°C. The residue was then re-extracted for 24 h with chloroform and methanol (chloroform : methanol : 1 N HCl; 5 : 2 : 3, v : v : v). After centrifugation at 650 × g for 30 min, the chloroform phase was recovered, evaporated to dryness, and methylated with 'magic' methanol (methanol : chloroform : concentrated HCL; 10 : 1 : 1, v : v : v) (W. Mayberry, personal communication) at 100°C for 1 h. The methylated hydroxy fatty acids (0HFA) were recovered in hexane:chloroform (4 : 1, v : v) in three washes, and the volatile solvent was then removed with a stream of nitrogen. The OHFA were further purified by thin layer chromatography using 250 μm layer LK6 silica gel plates (Whatman Limited Inc., UK). The plates were pre-cleaned and developed in hexane : diethyl ether (1 : 1, v : v). The OHFA band was recovered after location of an authentic OHFA standard, composed of α- and β-hydroxy myristic acid (Matreya, Inc., Pennsylvania), which had been chromatographed simultaneously. Before GC/MS analysis, the hydroxyl groups were derivatized with N, O-bis(trimethylsilyl)trifluoroacetamide. Hydroxy fatty acids were identified in the aquifer samples from the total ion trace by use of the extracted ion program. Confirmation of the hydroxy fatty acid structures was done using the VG-Trio 3 tandem mass spectrometer with positive ion detection after electron impact at 70 eV using the same temperature program (described above) with a Restek RT_x-5 capillary column (30 m × 0.25 mm i.d. and 0.1 mm film thickness).

Fatty acid nomenclature

Fatty acids were designated by the total number of carbon atoms: number of double bonds, with the position of the double bond closest to the methyl end (w) of the molecule. Configuration of the double bonds is indicated as *cis* (c) or *trans* (t). For example, 16 : 1w7c is a PLFA with 16 total carbons with one double bond 7 carbons from the methyl end in the cis configuration. Branched fatty acids are designated as *iso* (i) or *anteiso* (a) if the methyl branch is one or two carbons respectively from the w end (i15 : 0), or by the position of the methyl group from the carboxylic end of the molecule (10me16 : 0). Methyl branching at undetermined positions in the molecule is indicated as 'br'. Cyclopropyl (cy) fatty acids are designated by the total number of carbons (cy17 : 0). The position of the hydroxyl group is numbered from the carboxyl end of the fatty acid with OH as a prefix (3-OH16 : 0).

Statistical analysis

The PLFA profiles of the bacteria were treated as multivariate data and analyzed using hierarchical cluster analysis. Dendrograms were constructed using an incremental sum of squares method with the PC-based software package, Ein*Sight (Infometrix; Seattle, WA). Similarity values (1 = identical) were determined by the

Ein*Sight program using modified Euclidean distances.

Chemicals. Capillary grade GC/GC/MS solvents were obtained from Burdick and Jackson, (McGaw Park, IL). Reagents were purchased from Aldrich Chemical Co., (Milwaukee, WI), Sigma Chemical Co., (St. Louis, MO) and Pierce Chemical Co., (Rockford, IL).

Results

Fatty acid composition

The fatty acid composition derived from the PLFA and the LPS-OHFA of *D. tiedjei* grown with pyruvate alone, pyruvate and 3CBz, formate and 3CBz, and isovanillate and sulfate as electron donors and acceptors respectively is given in Table

Table 1

Fatty acid profiles of D. tiedjei cultivated under various conditions

PLFA (mol%)	#1	#2		#3		#4	
		x	S.D.	x	S.D.	x	S.D.
14:0	17.3	9.08	0.01	19.09	0.43	10.63	1.14
15:0	0.38	0	0	0	0	0	0
16:0	26.54	26.68	0.01	18.07	0.12	26.24	1.01
18:0	23.85	20.84	0.42	25.87	0.4	23.73	0.74
14:1	0.35	0.62	0	1.9	0.09	1.5	0.03
14:1w5c,t	0.97	0.33	0.02	1.21	0.08	0.33	0.47
16:1w9c	0.71	0.6	0.05	1.97	0.07	1.71	0.3
16:1w7c	23.7	18.63	0.06	15.81	0.08	20.48	1.84
16:1w7t	0.47	1.08	0	0.78	0.05	0.34	0.49
16:1w5c	2.06	2.07	0.05	1.89	0.05	2.51	0.06
cy17:0	1.4	15.52	0.01	4.7	0.13	7.16	0.37
18:1w7c	0.5	0.8	0	1.32	0.02	1.26	0.26
i15:0	0.67	0.34	0.08	1.32	0.04	1.13	0.49
Others [x]	0	3.41		6.07		2.98	
Total mol%	100	100		100		100	
cy17:0/16:1w7c	0.06	0.83	0.17	0.3	1.63	0.02	0.27
pmol PLFA/mg	9372	10538	942	9772	1077	6993	279
residue OHFA (mol%)							
3-OH16:0		18.11	2.59	20.83	7.17	20.05	6.18
3-OH18:0		9.17	0.88	9.95	1.97	11.82	1.74
br3-OH19:0a		3.07	0.11	4.53	0.78	3.77	1.31
br3-OH19:0b		4.65	0.41	13.96	2.63	11.47	1
br3-OH19:0c		2.29	0.06	6.05	1.22	4.25	0.53
br3-OH21:0		47.96	2.32	31.26	4.28	35.65	4.69
br3-OH22:0		13.23	0.82	9.35	1.19	9.65	1.15
Others [y]		1.52		4.07		3.34	
Total mol%		100		100		100	
pmol OHFA/mg	ND	918	88	1406	71	949	35
pmol OHFA/pmol PLFA		0.09		0.14		0.14	

[x] -Includes 12:0, 14:1, a15:0, 15:1's and 18:1w5c.

[y] -Includes 3-OH14:0 and br3-OH20's.

Fatty acid profiles of *D. tiedjei* grown under various conditions. Columns #1, #2, #3, and #4 are the profiles obtained when the isolate was grown with pyruvate fermentatively, with pyruvate as carbon source and 3CBz as electron acceptor, with formate as carbon source and 3CBz electron acceptor, and with isovanillate as carbon source and sulfate as electron acceptor, respectively. All cells were grown at 37°C and harvested in early stationary phase. The mol percent data is given as mean ± standard deviation, $n = 2$ except for the fermentatively grown cells where $n = 1$.

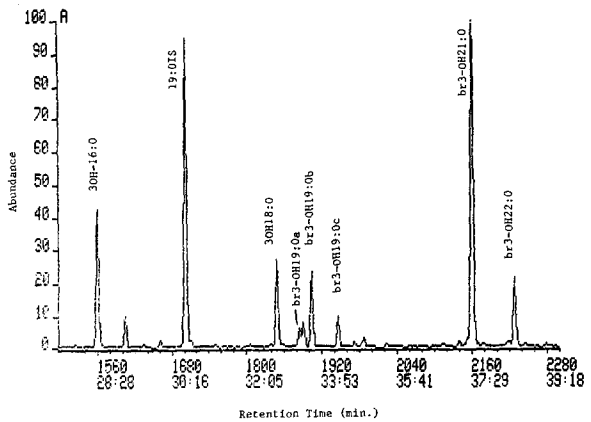


Fig. 1. Total ion chromatogram of the LPS-OHFA from *D. tiedjei*.

1. The total amount and proportions of PLFA and LPS OHFA are essentially the same and independent of growth conditions. The ratio of cy17:0 to 16:1w7c varies between 0.02 and 0.83 for the various growth conditions. Growth with pyruvate and 3CBz induces a higher proportion of cy17:0 than the other conditions. These cells also have a higher proportion of br3-0H21:0 and br3-0H22:0 and lower proportions of br3-0H19:0 in the lipid extracted residue than that measured in cells grown under other conditions.

The PLFA profiles from the three *D. tiedjei* cultures, grown with an external electron acceptor, were compared to PLFA profiles obtained from 25 species of sulfate-reducing bacteria. The three *D. tiedjei* cultures clustered together at a similarity index of 0.85, based on the incremental sum of squares method, and showed similarities to the sulfate-reducing bacterial species [13]. *D. tiedjei* has a PLFA profile that most closely corresponds to three groups consisting of *Desulfobacter latus* and *Desulfobacter curvatus* (similarity index = 0.79); *Desulfovibrio sapovorans* and *Desulfovibrio africanus* (similarity index = 0.80); and *Desulfosarcina variablis* and *Desulfococcus multivorans* (similarity index = 0.82) with a similarity index of 0.45.

Identification of the signature LPS-OHFA branched long chain fatty acids

Capillary GC/MS clearly shows three distinct branched 3-0H19:0 components (Fig. 1). Elution of these components prior to that of the normal

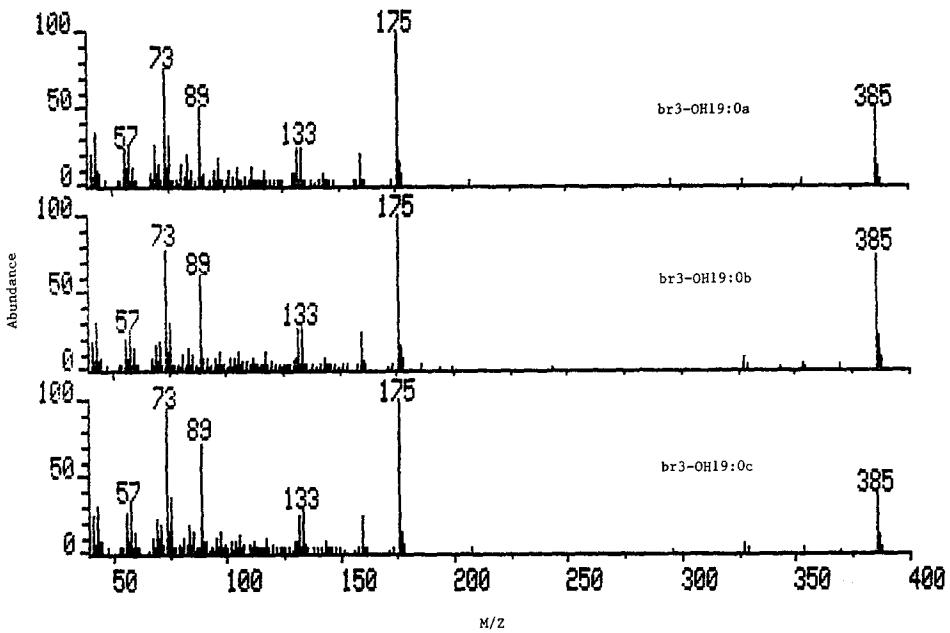


Fig. 2. Fragmentation pattern of the three br3-0H19:0 fatty acids from the LPS of *D. tiedjei*.

saturate implies branching. Each shows a m/z base peak of 73 and the 3-0H 'signature' at m/z 175. The ion at m/z 385 (M-15) indicates there to be 19 carbons in the molecule (Fig. 2). The 3-0H16:0, 3-0H18:0, br3-0H21:0 and br3-0H22:0 OHFA show M-15 peaks at 343, 371, 413, and 427 respectively. This pattern of LPS-OHFA has not been detected in other bacteria, and to our knowledge is unique to *D. tiedjei*.

Recovery of D. tiedjei lipids from inoculated aquifer slurries

Aquifer incubations containing between 57 and 290 pg PLFA (g dry wt)$^{-1}$ were inoculated with *D. tiedjei*, immediately frozen, and lyophilized. Analysis of the aquifer sediment mixtures yielded a number of PLFA that were not found in *D. tiedjei,* but were found in other microorganisms such as the 10 methyl branched 16:0 and 18:0 as well as i17:0, 17:1w6c, 18:2w6 and 18:1w9c. Analysis of the LPS-OHFA profiles by hierarchical cluster analysis showed ten of the sediment samples clearly grouped with the *D. tiedjei* cultures (Fig. 3). After analysis of the mixtures was complete, sample designations were decoded revealing that bottles with greater than 2×10^8 *D. tiedjei* cells clustered with and showed OHFA patterns similar to those of *D. tiedjei*, while all others did not. Cluster analysis of the PLFA patterns showed no consistent relationship between the inoculated samples and the *D. tiedjei* cultures. Comparison of the total PLFA g^{-1} and LPS-OHFA (g dry wt)$^{-1}$ showed consistently increasing values with increased level of *D. tiedjei*

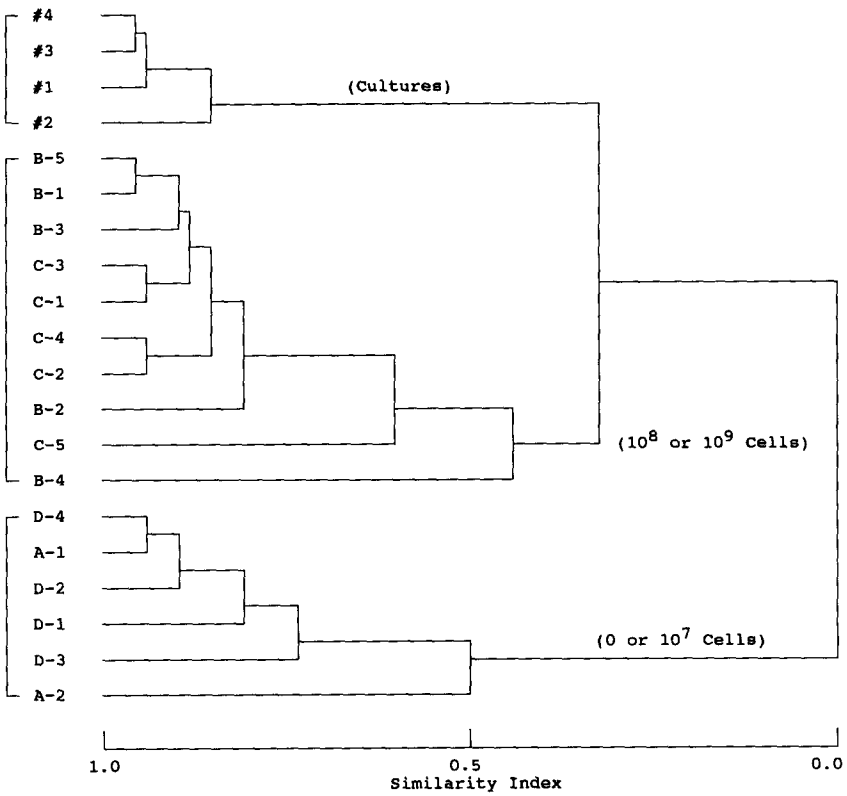


Fig. 3. Hierarchical cluster analysis indicating the cultures of *D. tiedjei* (Cultures), the aquifer samples amended with $> 2 \times 10^8$ *D. tiedjei* (10^8 or 10^9 cells) and the aquifer samples with no amendments or with $< 2 \times 10^8$ *D. tiedjei* (0 or 10^7 cells). Culture numerals refer to different growth conditions as in Table 1. A series inoculated with 2×10^7 cells, B series inoculated with 2×10^8 cells, C series inoculated with 2×10^9 cells and D series amended with sterile water.

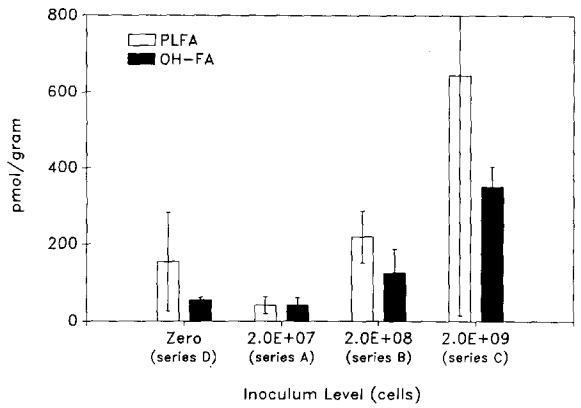


Fig. 4. Total PLFA (clear) and LPS-OHFA (dark) in pmol g^{-1} dry wt in the control aquifer sediment slurries (no cells) and in slurries amended with 2×10^7, 2×10^8 and 2×10^9 *D. tiedjei*. Series designation as in Fig. 3.

for additions of $> 2 \times 10^8$ cells (Fig. 4). Principal component analysis of the LPS-OHFA, which allowed detection of the inoculated samples, showed separation (with overlap) between the isolates, inoculated samples and uninoculated controls primarily based on the abundance of 3-OH16:0 and 3-OH18:0 (Fig. 5, principal component 1 loadings). A clear distinction between the sediments amended with 2×10^8 and 2×10^9 cells and those which either contained 2×10^7 cells or were uninoculated was apparent and dependent on the proportions of br3-OH21:0 and br3-OH19:Ob (Fig. 5, principal component 2 loadings).

Dehalogenating activity in the sediments

The effect of inoculation with *D. tiedjei* on 3CBz disappearance in aquifer sediment incubations amended with 0, 2×10^7, 2×10^8 and 2×10^9 cells of *D. tiedjei* as compared to a sterile control is shown in Table 2. All three levels of inoculum stimulated 3CBz degradation in the aquifer incubations; however, the inoculum size did not greatly influence the overall degradation of 3CBz. Although some stimulation in degradation could be attributed to increased inoculum size early in the incubation period, at later stages, little difference in the rates or extent of degradation could be detected.

Discussion

The analysis of extracted membrane lipids from environmental samples provides a quantitative measure of the viable microbial biomass, community structure and nutritional status of the microflora without requiring release of the organisms from the substratum or their subsequent cultivation [2,14]. Analysis of PLFA has been used to define the microbiota in deep subsurface sediments [15,16] including the identification of community shifts as a result of contamination [15–18]. Other applications of the technology have been in defining disturbance in marine sediments [19], in defining the nutritional status of the rhizosphere [20] and deep sea hydrothermal vent microbiota [21], as well as in compost treatments [2]. Analysis of LPS-OHFA from the lipid-extracted residue of environmental samples provides a means by which organisms containing unusual compositions of theses fatty acids can be detected. LPS-OHFA analysis has been used to detect Gram-negative organisms in marine and estuarine sediments [5]. An organism such as

Table 2

Concentration of 3CBz (μM) in aquifer incubations following [a]

Number of cells added	Day 0	7 weeks	14 weeks	20 weeks	30 weeks
2E+07	525±53	586±13	509±20	370±57	0
2E+08	541±57	543±81	497±56	321± 1	0
2E+09	539±41	467±15	434±30	356± 7	0
Zero	506±41	569±83	571±53	592±36	384±32
Zero/autoclaved	579±28	557±91	574±29	618±26	521±32

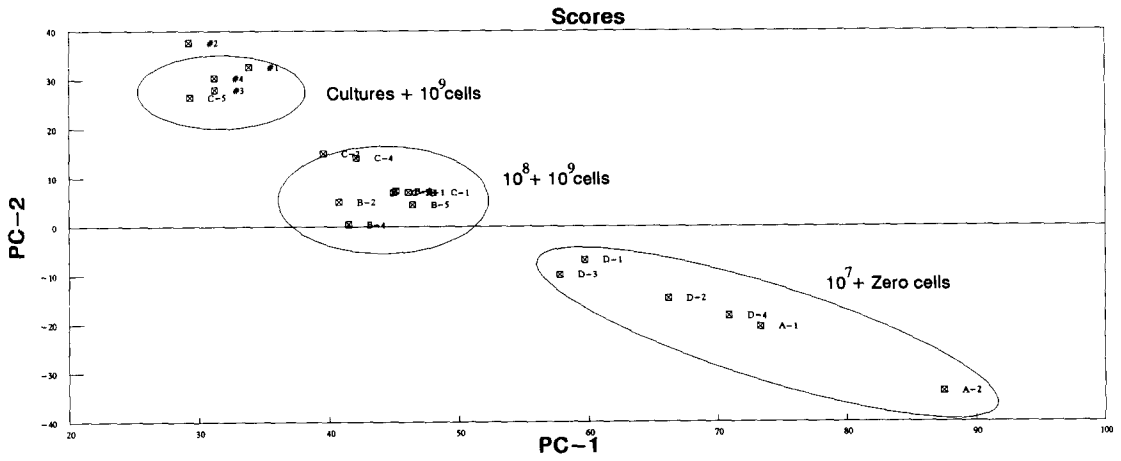
[a] Data is the average and standard deviation of three replicates.

Planctomyces, which contains unusual LPS-OHFA, could be readily detected using these methods [22]. LPS-OHFA, much like those from *D. tiedjei*, have been detected in anaerobic freshwater sediments and in digester samples (Ringelberg and White, unpublished data).

The PLFA of *D. tiedjei* were not sufficiently different from other sulfate-reducing bacteria to allow distinctions to be made between the three groups of sulfate-reducing bacteria, based on hierarchical cluster analysis [13]. Thus, *D. tiedjei* PLFA are not suitable to be considered signature biomarkers. The localization of LPS-OHFA and the patterns and structures of the long-chain branched LPS-OHFA of *D. tiedjei* are unique thus far in the microbial world and are satisfactory signature biomarkers. The differences in the patterns of PLFA or LPS-OHFA abundance as induced by growth with different substrates and terminal electron acceptors was minimal.

In this study, it was possible to consistently detect the presence of $> 2 \times 10^8$ cells of *D. tiedjei* in inoculated sediments based on the presence of signature long chain LPS-OHFA. Two LPS OHFA, br3-OH19:0 and br3-OH21 were the most significant of the branched 3-OHFA in the detection of *D. tiedjei* in sediments. The detection of anaerobes with long-chain-branched 3-OHFA in the lipid extracted residue from environmental samples could strongly suggest the presence of *Desulfomonile*-like sulfate-reducers.

The effect of inoculation with *D. tiedjei* on 3CBz degradation in simple aquifer incubations was examined. Although inoculation with *D. tiedjei* cells stimulated 3CBz degradation, the amount of degradation was not dependent on the size of the inoculum (Table 2). Similar results have been reported for the aerobic degradation of polycyclic aromatic hydrocarbons in simple laboratory microcosms [23,24].



Loadings

	PC-1		PC-2
3-OH16:0	0.875	br3-OH21:0	0.780
3-OH18:0	0.374	br3-OH19:0b	0.429
br3-OH19:0c	0.182	br3-OH22:0	0.180
br3-OH21:0	0.170	br3-OH19:0c	0.162
br3-OH19:0b	0.163	br3-OH19:0a	0.136
br3-OH19:0a	0.072	3-OH18:0	0.128
br3-OH22:0	0.032	3-OH16:0	-0.338

Fig. 5. Principal components analysis of the LPS OHFA derived from the amended and control aquifer samples. Classes and sample designations are given in Fig. 3.

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