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Phospholipid fatty acid and infra-red spectroscopic analysis of a sulphate-reducing consortium

Nicholas J.E. Dowling [a], Peter D. Nichols [b] and David C. White [a]

[a] *Institute for Applied Microbiology, University of Tennessee, Knoxville, Tennessee, U.S.A. and* [b] *CSIRO Marine Laboratories, Hobart, Tasmania, Australia*

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1. SUMMARY

In order to validate unusual fatty acids as biomarkers for sulphate-reducing bacteria, selective conditions were arranged for the enrichment of a marine glutamate-fermenting bacterium which made hydrogen and acetate available for oxidation via the respiration of sulphate. Under these conditions the complete oxidation of glutamate via sulphate reduction accounted for 84% of the available electron equivalents. Fatty acid biomarkers for hydrogen-oxidizing *Desulfovibrio* sp. (iso 17 : 1w7c and branched monoenoics) and for acetate-oxidizing *Desulfobacter* (10 methyl 16 : 0) were detected in the enrichment. These biomarkers were demonstrated in pure cultures of *Desulfovibrio* sp. and *Desulfobacter* sp. obtained from the enrichment. The predominant glutamate-fermenting bacterium isolated from the consortium contained no branched ester-linked phospholipid fatty acids, and produced acetate and hydrogen. With energy limitation the enriched consortium produced increased amounts of extracellular polysaccharide and the endogenous storage lipid poly-beta-hydroxybutyrate as detected with Fourier transform/infra-red (FT-IR) spectroscopy.

2. INTRODUCTION

Classical methods for describing microbial consortia often involve isolation or other disturbance of the community structure. One method of circumventing this problem has been the use of biomarkers [1]. These are unique or rare cellular components that may be used to differentiate some organisms in a mixture of others [2].

Specific biomarkers have been proposed for several types of sulphate-reducing bacteria [3,4,5] including the fatty acids 10-methyl hexadecanoate (10 Me16 : 0) and iso-heptadecen-9,10-oate (iso 17 : 1w7c) for *Desulfobacter* spp. and *Desulfovibrio* spp., respectively. These fatty acids have also been observed in several anoxic sediments [6,7,8]. The addition of acetate to marine sediment with the intent of stimulating the biomarkers of *Desulfobacter* spp. showed very little shift in fatty acid proportions (N. Dowling, unpublished data). This was almost certainly due to rapid mineralization of the substrate [9]. In this study a system was

Correspondence to: Nicholas J.E. Dowling, Institute for Applied Microbiology, Building 1, Suite 300, 10515 Research Drive, Knoxville, TN 37932, U.S.A.

designed to deliver low levels of substrate via microbial catabolism to target bacteria in a sediment enrichment. Thus one type of bacteria may be used to enrich others that possess signature biomarkers.

A fermenting bacterium was enriched with the intention of making acetate and hydrogen available for oxidation via specific sulphate-reducing bacteria (SRB). Fatty acid analysis of the consortium and individual members demonstrated the use of the proposed biomarkers. In a multistage flow system, conditions were arranged so that fatty acid distributions could be observed in substrate gradients. Bacteria in the flow system were also analysed for nutritional status by Fourier transform infra-red spectroscopy.

3. MATERIALS AND METHODS

3.1. Continuous flow system

A continuous flow system of four subsequent fermenters of 800 ml operating volume each, was set up, kept at 20 °C and supplied by a 12 l sterile medium vessel. The dilution rate was adjusted to 0.1 h^{-1}. The system was constantly gassed with N_2/CO_2 (9 : 1) at approximately 15 ml/min. The total run time was over 2000 h. Periodic analyses were performed to determine sulphate, sulphide, glutamate, and volatile fatty acid concentrations. Numbers of free cells in the bulk phase were determined by acridine orange direct counts [10]. The flow system was inoculated with 10 ml of black sulphide-rich marine sediment obtained from Turkey point, Florida.

3.2. Media

Saltwater medium was used for these experiments which contained (g/l) Na_2SO_4 3.0, KH_2PO_4 0.2, NH_4Cl 0.25, NaCl 20.0, $MgCl_2$ 4.0, KCl 0.5, $CaCl_2$ 0.15, and 0.5 mg/l resazurin as redox indicator. Complex vitamins included biotin, folic acid, pyridoxine HCl, thiamine HCl, riboflavin, nicotinic acid, cyanocobalamine, *p*-aminobenzoate, lipoic acid, and pantothenic acid, Na_2S as reductant, trace elements and selenite solution were added [11]. The buffer was provided by $NaHCO_3$ (30 mM) and CO_2. The final pH was adjusted to 7.0 to 7.4 in all cases. Glutamate (10 mM) was supplied as organic carbon source in the continuous flow system and during isolation and cultivation of glutamate fermenting bacteria. SRB were isolated with acetate (10 mM), lactate (10 mM), and H_2/CO_2 (20 : 80) + acetate (5 mM). Media used for the isolation of fermenting bacteria contained no sulphate. Both SRB and the glutamate fermenters were isolated with the agar 'shake-tube' method of Pfennig and coworkers [12] and employed 1% agar. Detection of intracellular poly beta-hydroxybutyrate granules was by Sudan black staining [10]. Pure cultures for lipid analysis were grown in duplicate.

3.3. Chemical analyses

Sulphate and sulphide were assayed in triplicate using the methods of Tabatabai [13] and Cline [14], respectively. Glutamate was first desalted on Bio-Rad AG 50W-X8 (H^+) 'Econo-columns' (Bio-Rad, Richmond, CA) after Harris et al. [15] and then assayed using the Waters Associates Inc. HPLC Pico-Tag method. Volatile fatty acid analysis was carried out on aqueous samples acidified with formic acid (1%). The assay used a Packard model 419 gas chromatograph fitted with a Supelco SP1200 packed-column and flame ionisation detection. Hydrogen and dissolved carbon dioxide levels were assayed using a Packard model 417 fitted with a thermal conductivity detector. Carbon dioxide calibration curves were generated by acidifying known amounts of $NaHCO_3$ in closed 60 ml bottles and then sampling the headspace. Desulfoviridin was assayed in all bacteria by centrifuging 10 ml of culture, resuspending the pellet in 2 M NaOH and checking for red fluorescence under ultraviolet light [16].

3.4. Fourier transform / infra-red spectroscopy

Analyses were carried out as described previously [17,18] with a Nicolet 60SX FT/IR. Biofilm areas of approximately 2 cm^2 were scraped off the inside of each pot shortly after dismantling the continuous flow system. These were freeze-dried, then pulverised for 1 min in a Wig-L-Bug (Spectra Tech, Inc., Stamford, CT) with powdered KBr (sample/KBr, 1 : 10) and analysed as such. FT/IR of the samples was carried out by diffuse reflec-

tance (Drift) with a resolution of 8 cm^{-1} at wavenumbers 800–4000 cm^{-1}. Detection was with a liquid nitrogen cooled mercury/cadmium/tellurium (MCT) high sensitivity detector.

3.5. Lipid analysis

All samples were extracted using a Bligh and Dyer one phase mixture of chloroform/methanol/3% aqueous NaCl (37.5 : 37.5 : 30 ml). The fatty acids were then separated and methylated as previously described [6]. The free cell material in the bulk phase of the continuous flow system was recovered carefully by pouring out the liquid of each pot into clean lipid-free polypropylene bottles and centrifuging. The biofilms were directly extracted from each pot by swirling the extraction mix. Phospholipid ester-linked fatty acids were identified by gas chromatography (GC) retention time and gas chromatography-mass spectrometry (GC-MS) of the original fatty acids, their dimethyldisulphide adducts [19] and hydrogenated derivatives [6]. The biofilms and free cell material were also examined for total, ester-linked and ether-linked lipid phosphate [20]. GC and GC-MS were carried out as described previously [6].

4. RESULTS

4.1. Continuous flow system

After over 2000 h operation the flow system was assayed for substrate changes. The final values obtained are presented in Fig. 1. Results show that glutamate was oxidised to acetate and then to carbon dioxide at the expense of sulphate which was in turn reduced to sulphide. Hydrogen was not detected by packed column GC in any of the pots. Dissolved carbon dioxide (not shown in Fig. 1) increased from 20 mM in the medium vessel to 63 mM in pot P4. The levels of sulphide did not increase stoichiometrically (0.4–5.2 mM) with the decrease in sulphate concentrations (21.0–2.10 mM) probably due to the stripping effect by the headspace gas. The only volatile fatty acid detected was acetate in P1 at 2.2 mM.

4.2. Isolation and characterization of bacteria

The continuous flow system was examined for the presence of bacteria which together performed the complete anaerobic oxidation of glutamate.

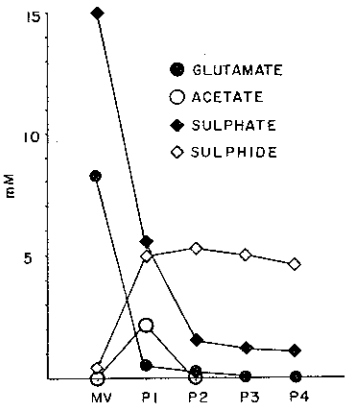

Fig. 1. Substrate changes in the continuous flow system assayed in the media vessel (MV) and pots P1–P4 where P1 had sterile media introduced whereas all other pots were fed the waste from the previous vessel.

A Gram-negative, non-motile anaerobic glutamate fermenting rod-shaped bacterium was isolated which produced acetate, butyrate, carbon dioxide and hydrogen as major products. Old cultures of this fermenting bacterium showed cellular inclusions which stained positive for polyhydroxybutyrate. No spores were detected. This bacterium was desulfoviridin-negative.

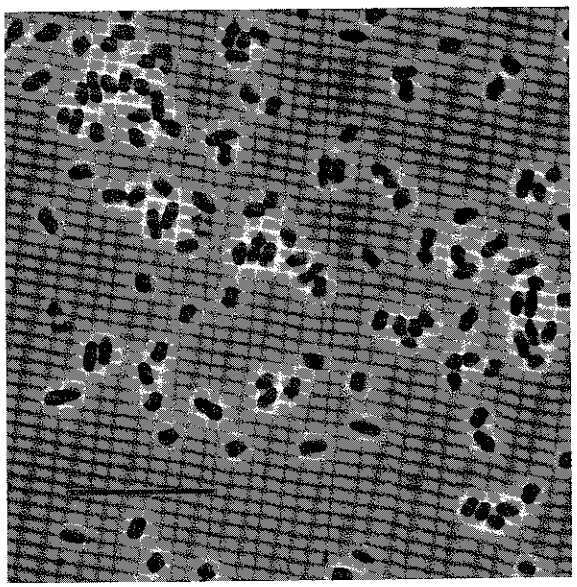

Fig. 2. Phase-contrast micrograph of the *Desulfobacter* isolate. Bar represents 10 μm for Figs. 2 and 3.

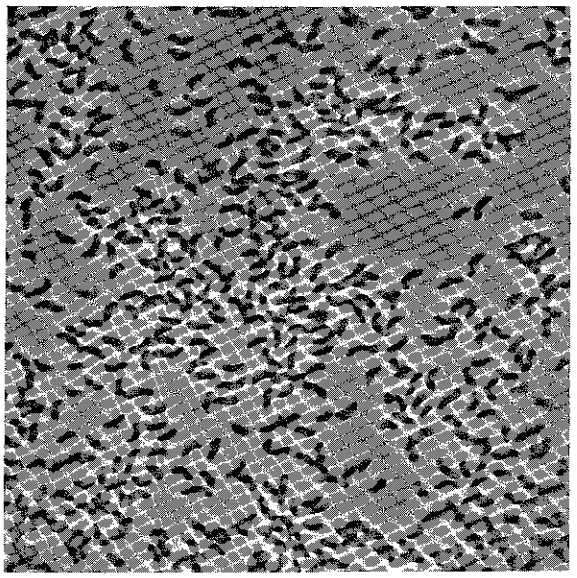

Fig. 3. Phase-contrast micrograph of a *Desulfovibrio* isolate.

An acetate-oxidizing sulphate-reducing bacterium was isolated which was oval to rod-shaped, non-sporing, desulfoviridin-negative and non-motile. This isolate could not utilize propanoate, lactate, hydrogen, or formate as electron sources. Ethanol was utilized very slowly. This bacterium was assigned to the genus *Desulfobacter* (Fig. 2) according to the scheme by Widdel and Pfennig [11].

A hydrogen-oxidizing sulphate-reducing vibrio (sometimes spirillar) was isolated which used carbon dioxide and acetate as carbon sources. This SRB was highly motile and desulfoviridin-positive. A very similar sulphate-reducing vibrio was isolated with exactly the same characteristics as above which oxidised lactate. Both SRB could grow with lactate or hydrogen and were assigned to the genus *Desulfovibrio* (Fig. 3).

4.3. Lipid analysis

All the extractable lipid phosphate (LPO4) from the continuous flow system occurred in the ester-linked form. No ether-linked lipids were detected. Thus no methanogenic or plasmalogen-containing bacteria contributed significantly to the biomass of the system. The quantity of LPO4 in each pot is presented in Fig. 4. The majority of the biomass in every pot, (expressed as phospholipid phosphate, [20]) was located in the biofilm attached to the glass wall of the vessel. As the substrate became limiting with increasing pot number (P1–P4) the biofilm LPO4 decreased from 39 μmol LPO4 in pot P1 to 11.5 μmol LPO4 in pot P4. The free cell LPO4 also decreased in the same direction from 2 to 1 μmol phosphate. The slight increase in the free cell LPO4 in pot P3 was due to a floc of bacteria detaching from the biofilm when pouring out the bulk phase. The results of the acridine orange counts of the bulk phase (free cell material) show that the total cell numbers in the pots decreased from 2.5×10^{13} to 0.4×10^{13} as the pots became increasingly energy limited.

Fatty acid analysis of the continuous flow system showed that the proposed biomarkers 10Me16 : 0 and iso 17 : 1w7 occurred in every vessel biofilm and bulk phase. The fatty acid iso 17 : 1w7c comprised between 0.9% and 0.6% of the biofilm fatty acids, and 0.7% to trace amounts in the bulk phase. The unattached (free cells) bacteria were different in composition to those associated with the biofilm. The 10-methyl fatty acids comprised 15.1% to 18.8% of the biofilm fatty acids and 1.9% to 9.5% of the free cell fatty acids.

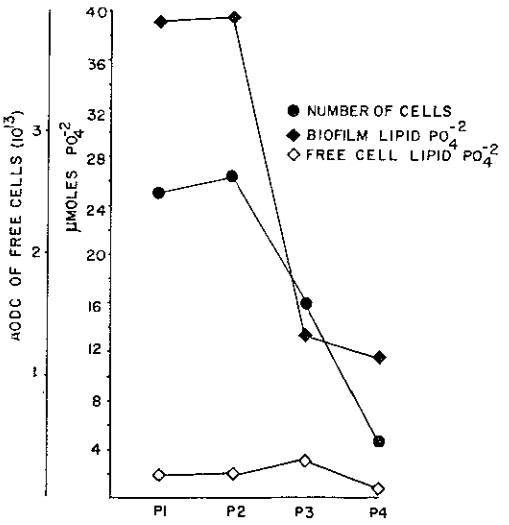

Fig. 4. Total lipid phosphate in each pot (P1–P4) of the continuous flow system located in the biofilm and free cells in the bulk phase. Acridine orange direct counts of the free cells are also included.

Table 1

Phospholipid ester-linked fatty acids of the biofilm (BF1) and free cell material (FC1) from pot 1 of the continuous flow system. Values given are expressed as percentages of the total fatty acids

FAME	BF1	FC1	FAME	BF1	FC1
12:0	0.2	–	10Me 16:0	14.4	2
iso 13:0	0.2	–	iso 17:0	0.3	0.1
iso 14:0	0.2	tr	a 17:0	0.1	0.1
a 14:0	tr	–	17:1w8	0.5	0.2
14:1w7	0.1	–	17:1w6	0.7	0.3
14:1w5	0.8	0.6	cy 17:0a	10.9	1.3
14:1 *	0.2	0.2	cy 17:0b	0.6	–
14:0	10.6	6.5	17:0	0.7	0.6
br 15:1w7 *	0.4	tr	br 18:0 *	0.2	–
iso 15:0	4.2	1.6	br 18:1 *	0.3	–
a 15:0	0.7	0.9	10Me 17:0	0.4	tr
15:1 *	0.1	tr	18:1w9	1.2	3
15:1w8	0.2	tr	18:1w7	3.3	6.2
15:0	1.8	0.8	cy 18:0a *	0.3	–
iso 16:1w6	0.1	–	cy 18:0b *	0.5	tr
10Me 15:0	0.2	tr	18:0	0.8	0.8
iso 16:0	0.2	0.1	br 19:1w7 *	0.4	–
16:1w9c	3.5	11.7	10Me 18:0	0.1	–
16:1w7c	8.7	21.8	19:1a *	0.1	1.2
16:1w7t	0.4	1.0	19:1b *	0.1	–
16:1w5	1.7	1.5	cy 19:0	1.6	0.2
16:0	25.1	35.6	Other FAME	2.0	1.2
iso 17:1w7c	0.9	0.5			

* Indicates that the substituent nature or position is uncertain FAME-fatty acid methyl ester. br-branched. cy-cyclopropyl. iso-penultimate methyl branch from the methyl end of the fatty acid. a-anteiso methyl branch. 18:1w6 indicates an 18-carbon length chain with one position of unsaturation at the 6th carbon from the methyl end.

Cyclopropyl fatty acids were 8% to 12.3% in the biofilm while only 1.3% to 5.8% in the free cells. Monounsaturated fatty acids were 19.1% to 23.7% of the biofilm but 34.7% to 50.5% of the free cells. Straight chain (all unbranched) fatty acids were 29.2% to 61.1% of the biofilm and 72.8% to 92.6% of the free cells. No other major differences in the flow system fatty acid composition were detected. The fatty acids of the biofilm material and of the free cell material of pot P1 are listed for comparison in Table 1. The fatty acid compositions of the various isolates differed widely (Table 2). The glutamate fermenter exhibited only straight chain (100%) and monoenoic (63.8%) fatty acids. No branched fatty acids were detected. The major fatty acid was 17:1w8.

The *Desulfobacter* sp., as expected, contained high quantities of 10Me16:0 (16.4%) and cy 17:0 (4.31%) with both monounsaturated (35.8%) and branched (28.1%) fatty acids. The major acid was 16:0.

Both the *Desulfovibrio* spp. isolated contained iso 17:1w7 in significant quantities (lactate culture 5%, H_2/CO_2 culture 6.4%) and had very similar fatty acid compositions. The lactate isolate contained 42.1% unsaturated to 60.9% branched acids while the H_2/CO_2 isolate had 39% and 41.5%, respectively.

4.4. FT/IR spectroscopy

FT-IR data for the small quantities of biofilm obtained from the continuous flow system are presented in Fig. 5. This shows the energy absorption in the infra-red region due to various chemical bonds. The broad band with a maximum at 3300 cm^{-1} is due to the hydroxyl (O–H) and N–H stretching. At 1750 cm^{-1} (seen best with the resolved peak in BF4) is the carbonyl (C=O) ab-

Table 2

Phospholipid ester-linked fatty acids of four anaerobic bacteria isolated from the continuous flow system. Values given are average percent values of the total fatty acids for two duplicate cultures

FAME	Glutamate fermenter	*Desulfobacter* sp. (Acetate)	*Desulfovibrio* sp. (H_2/CO_2)	*Desulfovibrio* sp. (Lactate)
11:0	0.41	–	–	–
12:0	0.15	–	–	–
13:1	1.20	–	–	–
13:0	3.03	–	–	–
iso 14:0	–	0.08	1.78	2.64
14:1a	0.69	–	–	–
14:1b	0.15	–	–	–
14:0	1.80	5.49	0.60	0.27
iso 15:1w7	–	–	0.25	0.17
a 15:1w7	–	–	0.18	0.16
iso 15:0	–	6.12	4.28	2.37
a 15:0	–	0.20	10.76	12.72
15:1w8	1.05	0.10	–	–
15:1w6	6.88	0.12	0.19	0.48
15:0	8.62	1.10	0.43	0.69
10Me 15:0	–	0.08	–	–
iso 16:1w6	–	–	3.48	7.83
iso 16:0	–	0.50	3.96	6.79
16:1w9	3.12	1.75	0.05	0.11
16:1w7c	7.81	18.61	9.30	4.37
16:1w7t	–	–	0.20	0.05
16:1w5	0.33	2.57	0.19	0.11
16:0	7.49	26.88	12.27	6.18
iso 17:1w7	–	1.59	5.07	6.36
a 17:1w7	–	–	2.74	5.18
10 Me 16:0	–	16.41	–	–
iso 17:0	–	0.98	1.58	1.74
a 17:0	–	0.13	3.31	7.32
17:1w8	26.50	0.46	–	–
17:1w6	7.37	1.57	1.05	3.74
cy 17:0	–	4.31	–	–
17:0	13.89	0.43	4.41	9.02
br 18:0	–	0.18	–	–
iso 18:1w8 *	–	–	0.20	0.53
iso 18:1w6	–	–	1.64	3.72
10Me 17:0	–	0.35	–	–
iso 18:0	–	–	1.08	1.46
18:1w9	5.41	0.60	0.49	0.29
18:1w7	2.20	6.86	12.75	7.98
18:1w5	–	–	0.27	0.18
18:0	0.72	0.45	15.82	5.55
iso 19:1w7	–	–	0.46	0.44
a 19:1w7	–	–	0.19	0.39
br 18:1	–	1.61	–	–
10Me 18:0	–	tr	–	–
iso 19:0	–	–	0.15	0.50
a 19:0	–	–	0.19	0.46
19:1a	0.30	–	–	–
19:1b	0.34	–	0.15	–
19:1c	0.44	–	–	–
cy 19:0	–	0.31	–	–

(*continued*)

Table 2 (*continued*)

FAME	Glutamate fermenter	*Desulfobacter* sp. (Acetate)	*Desulfovibrio* sp. (H_2/CO_2)	*Desulfovibrio* sp. (Lactate)
iso 20:1w6	–	–	0.02	0.03
iso 20:0	–	–	0.18	0.07
20:1	–	–	0.15	0.02
20:0	–	–	0.17	0.05
Unsat. FAME	63.79	35.84	39.02	42.14
Branched FAME	0.00	28.15	41.50	60.88

For fatty acid nomenclature and substituent abbreviations see Table 1.

sorbance arising from PHB (17), and at 1650 and 1550 cm^{-1} are the amide I and II peaks, respectively. The large absorbance centred at 1150 cm^{-1} is the carbohydrate-derived C–O stretch. While the ratio of hydroxyl to amide absorbance remains relatively constant for the four biofilm samples, changes were apparent in the relative band intensities of the PHB carbonyl and carbohydrate-derived C–O stretches (Fig. 5). The PHB-derived carbonyl C=O stretch is present as an unresolved shoulder in P1, P2 and P3. In P4, however, the C=O band is larger in intensity and is clearly resolved from the amide I band. Similarly, differences are apparent in the carbohydrate C–O (1150 cm^{-1}) to amide I (1650 cm^{-1}) ratio. A steady increase in this ratio is observed, indicating that as substrate becomes limiting (P1–P4) the quantity of glycocalyx increased relative to protein.

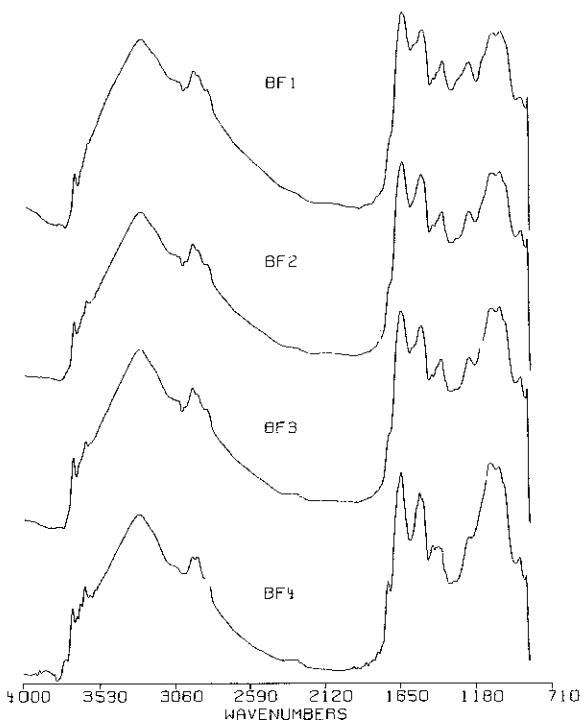

Fig. 5. Infra-red absorbance between 710–4000 cm^{-1} for the continuous flow system biofilms. Biofilms BF1–BF3 correspond to pots P1–P4.

5. DISCUSSION

In a continuous flow system glutamate was completely oxidized to CO_2 with concomitant reduction of sulphate to sulphide. Three bacteria were shown to predominate in this mixed bacterial community: a fermentative bacterium similar to *Acidaminobacter hydrogenoformans* [21], an acetate-oxidizing *Desulfobacter* spp., and a hydrogen-oxidizing *Desulfovibrio* type. Complete oxidation of the 10 mM glutamate provided would generate 180 mmol electron equivalents. The reduction of 18.9 mmol of sulphate required 151 mmol electron equivalents. Thus 84% of the glutamate was oxidized at the expense of sulphate.

Lipid analysis of the continuous flow system showed most of the biomass (as measured by lipid phosphate) to be associated with the biofilm and all of it to be eubacterial. Adhesion to surfaces is a well known bacterial response in minimizing the effect of substrate limitation [22]. The biofilm biomass, as measured by total lipid phosphate, decreased considerably with pot number (P1–P4) while the free cell material decreased only slightly. AODC enumeration of the free cell numbers con-

firmed the decrease. The reduction in activity (as measured by the difference in substrate concentration between pots) is to some extent mirrored by the decrease in biofilm biomass (total lipid phosphate Fig. 4).

Phospholipid ester-linked fatty acid analysis of the continuous flow system confirmed that a significant difference existed between the free cell community and that of the biofilm. Monoenoic fatty acid levels were higher in the free cell community than the biofilm, while the reverse was true for midchain branched and cyclopropyl fatty acids. A possible explanation is that the bacteria in the biofilm needed to retain the membrane flexibility imparted by double bonds, but in a protected form. Mid-chain branched (e.g. 10-methyl) and cyclopropyl substituents are just such modifications of monoenoic fatty acids [23,24]. An alternative explanation is that the bacteria are distributed differently with more *Desulfobacter* sp. in the biofilm and thus contributing more biomarker fatty acids to the total amount. No polyenoic fatty acids were detected which indicated the absence of eukaryotic organisms. Fatty acids associated with *Desulfobacter* spp. (10 Me16 : 0, 10Me 17 : 0, and cy 17 : 0) and *Desulfovibrio* spp. (iso 17 : 1w7, br 15 : 1w7, and br 19 : 1w7) were detected in every pot. The substituents (br) in the flow system were iso or anteiso branches. The failure to detect anteiso 17 : 1w7 in the continuous flow system and low levels of iso 17 : 1w7 among other characteristic fatty acids may indicate one of two events: 1) these fatty acids did not represent a high proportion of the total in the *Desulfovibrio* when they were in the system (unlike the proportions observed in pure culture). 2) *Desulfovibrio* spp. with these biomarkers did not represent a significant proportion of the community.

The glutamate fermenter was shown to have only straight chain fatty acids. Both saturated and unsaturated acids were detected. The lack of branched components provides an excellent distinction from the other isolates.

FT/IR analysis of the continuous flow system by diffuse reflectance spectroscopy (Fig. 5) showed an increasing carbohydrate (C–O) to protein/peptidoglycan (amide) ratio as substrate concentrations decreased. This shows that the glycocalyx levels in the biofilm increased significantly as substrate became limiting and the total biomass decreased. An absorbance in the last pot (P4) at 1750 cm^{-1} corresponded to the carbonyl stretch which is characteristic of PHB. Small absorbances on the shoulders of the amide I peak at the same wavenumber in pots P1–P3 may also be attributed to PHB. The only isolate containing PHB was the glutamate fermenter in stationary phase. This study has shown that the fatty acid biomarkers of *Desulfobacter* spp. (10Me16 : 0, cy 17 : 0) and *Desulfovibrio* spp. (iso 17 : 1w7, iso 15 : 1w7, and iso 19 : 1w7) may indeed be useful in characterizing hydrogen- and acetate-oxidising sulphate-reducing systems. Fourier transform/infra-red spectroscopy was also useful in characterizing the degree of carbon limitation. The increase in glycocalyx/protein + peptidoglycan ratio and PHB synthesis was demonstrated as substrate decreased. This information may prove useful as an indicator of nutritional status in further studies.

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