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DIFFERENTIAL CORROSION RATES OF CARBON STEEL BY COMBINATIONS OF *BACILLUS* sp., *HAFNIA ALVEI* AND *DESULFOVIBRIO GIGAS* ESTABLISHED BY PHOSPHOLIPID ANALYSIS OF ELECTRODE BIOFILM

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Abstract—The corrosion rates of carbon steel by monocultures and various combinations of aerobic (*Bacillus* sp.), fermentative (*Hafnia alvei*) and sulfate-reducing (*Desulfovibrio gigas*) bacterial biofilms in an aerobic, continuously flowing freshwater reactor containing 0.4 mM sulfate showed marked differences. Biofilm formation and electrode colonization resulted in decreases in the open circuit potential (OCP). The corrosion rate was measured non-destructively as the admittance ($1/R_{ct}$) by electrochemical impedance spectroscopy (EIS) on a four sided working electrode which allowed estimates of the reproducibility. The monocultures each induced greater corrosion initially but with time the rates of corrosion decreased to that of the sterile control. The *D. gigas* was unable to grow in the bulk phase but formed an apparently non-living biofilm. The admittance (the measure of corrosion) was greatest when the sulfate-reducing bacterium, *D. gigas*, was present in a consortium. The consortia containing *D. gigas* + *H. alvei* showed a significantly higher corrosion rate than the triculture or the other dicultures. The microbial biomass and the bacterial community structure actually on the electrode surfaces were examined by viable counts, most probable number (MPN) estimations and phospholipid fatty acid (PLFA) profiles determined after extraction and gas chromatographic analysis. The rate of corrosion was not directly related to the total microbial biomass or the number of species on the coupon. The rate of corrosion did not depend on the ratio of heterotrophic to sulfate-reducing bacteria (SRB) or absolute number of SRB. The PLFA analysis showed the organisms on the working electrodes were more stressed/starved than when grown in the bulk phase for inocula. This study demonstrates that different combinations of bacteria growing on the same substrata (electrode) in the same bulk phase induce very different corrosion rates. The maximum corrosion rate with the isolate combinations was within 70% of an enrichment with a community composition (determined by PLFA analysis) like that of the native corrosion tubercules.

INTRODUCTION

IN THIS study the effects of monocultures and different combinations of microbes colonizing the surface of mild steel in the same bulk phase in a flowing system will be shown to exhibit different rates of corrosion. Several mechanisms by which microbial metabolism in biofilms could effect corrosion have been postulated. Booth and Tiller suggested that the increased corrosion induced by biofilms of hydrogenase containing sulfate reducing bacteria (SRB) was caused by consumption of hydrogen at the cathode and postulated a cathodic depolarization mechanism.[1] Cathodic depolarization has also been associated with the activities of methanogenic bacteria.[2] A *Pseudomonas* sp. was shown to accelerate corrosion with the addition of a carbon source suggesting bacterial activity correlated with microbially influenced corrosion

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(MIC).[3] Other MIC mechanisms investigated have been organic acid production from fermentation by *Clostridia*[4] and iron reduction by an iron reducing bacterium.[5] MIC studies have demonstrated the effects of consortia composed of physiologically different bacteria. A diculture of *Vibrio anguillarum* and *Desulfovibrio vulgaris* produced a corrosion rate greater then either organism in monoculture.[6] Consortia of bacteria are often associated with MIC under *in situ* conditions.[7] SRB have also been shown to be active in anaerobic niches within a biofilm of aerobically respiring bacteria on metals.[7] SRB may show MIC activity in systems in which the bulk phase is aerobic.

In this study, mono-, di- and tricultures of *Bacillus* sp. (B) (an obligate aerobe), *Hafnia alvei* (H) (a facultative fermentative bacteria), and *Desulfovibrio gigas* (D) (ATCC #19364) (an SRB) were used to compare the effects of monocultures and different combinations of bacteria on the corrosion of carbon steel. Does the metabolic diversity of combinations of bacteria increase the potential for MIC? These combinations were chosen to compare the effects of bacteria capable of producing oxygen concentration cells, organic acids, reduced sulfur compounds and cathodic depolarization for the potential to induce corrosion.

EXPERIMENTAL METHOD

Bacterial media

The medium used for the isolation and subsequent inoculation of the corrosion flasks for the strictly aerobic B and the fermenting H contained, in g l^{-1}: glucose, 2; lactic acid, 2; NH_4Cl, 0.5; KH_2PO_4, 0.1; and $MgSO_4 \cdot 7H_2O$, 1, at pH 7. "Mineral salts" refers to the above media without the glucose and lactate. The media for culturing the sulfate-reducing D inoculum was, in g l^{-1}: lactic acid, 3; yeast extract, 1; $MgSO_4 \cdot 7H_2O$, 4; $CaSO_4$, 1; NH_4Cl, 1; and K_2HPO_4, 0.5. This media was supplemented with 1 g l^{-1} of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ for the most probable number test. The presence of a black precipitate, FeS, indicates growth of the organism. The medium for the corrosion experiments contained, in mg l^{-1}: glucose, 50; lactic acid, 50; KH_2PO_4, 5; NH_4Cl, 15; and $MgSO_4 \cdot 7H_2O$, 80.

Isolation and characterization of bacteria

The B and H were isolated from a carbon steel pipe tubercle and identified by their fatty acid patterns with the Microbial Identification System (MIS) (MIDI, Newark, DE). D was purchased from the American Type Culture Collection (Rockville, MD). The bacteria were grown in broth for 24 h at 25°C, centrifuged and washed twice with reduced mineral salts prior to inoculation. Viable counts for B and H were made on the glucose + lactate medium containing agar and could be readily distinguished by their colonial morphology.

MIC test system

The corrosion rates of the monocultures and combinations of bacteria were performed in a flow-through system constructed from a 1000 ml glass kettle closed at the top with a Viton O-ring seal and a polypropylene disk 1 cm thick (Fig. 1). The disk was clamped to the glass kettle and leads for the multiple sided working electrode (WE), the Luggin probe with a saturated calomel electrode (SCE) with a vicor tip as a salt bridge, the titanium counter electrode (CE), the inoculation port, the media inlet drip tube, ventilation port, and the exit port were positioned. The inlet port and drip tube were maintained under positive pressure from the sterile medium container. The ventilation port (through which air was sparged) and drip tube inlet were connected to filter holders containing 0.2 μm pore diameter filters. A magnetic stirrer mixed the test system. The level of medium maintained by positioning the medium exit tube which was connected to a peristaltic pump and a waste container. The entire apparatus was sterilized with ethylene oxide/carbon dioxide under carefully controlled humidity and dried in an oven to prevent corrosion of the mild steel. The total solution volume was 550 ml. The input flow rate was 1 ml min^{-1} and was controlled with a peristaltic pump using C-flex tubing (Cole-Parmer, Chicago, IL).

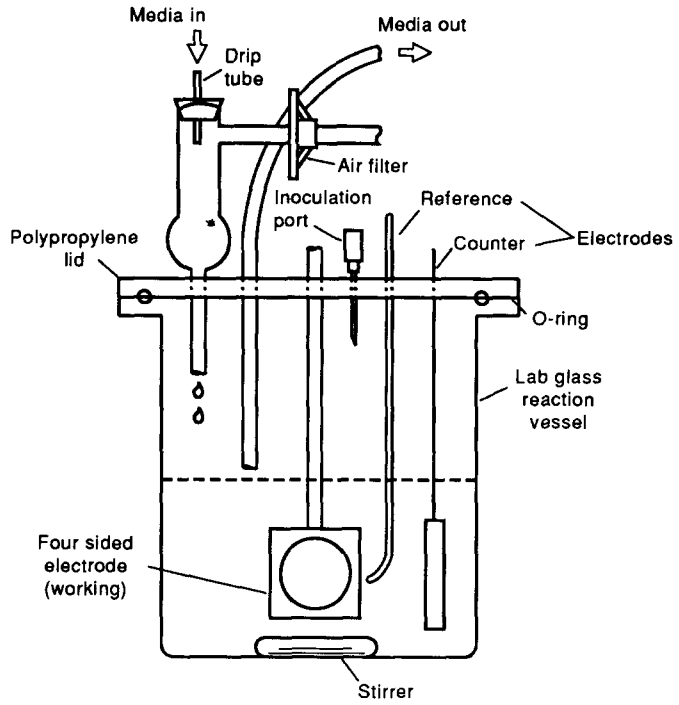

FIG. 1. The flow-through test system with multiple sided working electrode, counter electrode and Luggin probe.

Electrode preparation

Circular, 1 cm C1020 carbon steel coupons were used in all experiments (Metal Samples Co., Mumford, AL; composition in %; carbon, 0.17; manganese, 0.42; phosphorus, 0.009; sulfur, 0.006; and iron, 99.495). Four coupons were placed in a cube and then assembled together and embedded in epoxy (Acme, New Haven, CT) to create a four-sided electrode. The electrodes were then polished with 600 grit sand paper to a smooth finish, rinsed in acetone, distilled water and carefully dried with tissue. The electrode edges were then covered with Microshield lacquer (Pyramid Plastics, Hope, AK) and dried for 1 h. Each carbon steel face of the multiple sided WE has been shown to be independent of the others and could be rotated in the apparatus for optimal geometry relative to the Luggin probe for EIS measurement.[8]

Bacterial biofilm analysis

Upon completion of each experiment, the four sided electrode was aseptically removed and analysed for bacterial biomass. A glass tube just larger than the WE was clamped to the WE with an O-ring seal and the organisms recovered in the medium by gentle sonication for viable counting, or extracted directly with the one-phase lipid extractant for phospholipid ester-linked fatty acid (PLFA) analysis.[8,9] Two sides of the WE electrodes were used to analyse the viable biomass and community structure by PLFA profiles. The remaining two sides of the electrode were used for viable counts for the B and H and most probable number (MPN)[10] for D.

Phospholipid fatty acid extraction

The extraction, derivatization and quantification of PLFA has been described in detail.[11] The PLFA patterns defined in mol% of each fatty acid recovered from the polar lipids from each bacterial community were then clustered by complete linkage, farthest neighbor method with the mainframe software package SPSS-X (version 3.0). This allowed quantitative comparison of the total community structure recovered from the WE.

Fatty acid nomenclature

Fatty acids are 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 seven carbons from the methyl end in the *cis* configuration. Fatty acids that are branched are designated as *iso* (i) or *anteiso* (a) if the methyl branch is one or two carbons from the w end (i15:0). Cyclopropyl (cy) fatty acids are designated by the total number of carbons (cy17:0).

Electrochemical analysis

The open cell potential (OCP) was determined using a Solartron voltmeter model 7081 with a 7010 Minate scanner (Solartron-Schlumberger, Farnborough, U.K.) interfaced with a Hewlett Packard computer. AC impedance was determined using a 1286 Electrochemical Interface with a 1250 Frequency Response Analyzer (Solartron-Schlumberger, Farnborough, U.K.) controlled by a Hewlett Packard series 300 computer. A 5 mV r.m.s. sinusoidal signal was applied around the OCP from 10 Hz to 5 mHz for five cycles for five steps decade^{-1}. The data were plotted in complex plane (Nyquist) plot format and the solution resistance (R_Ω), polarization resistance or charge transfer resistance (R_{ct}) and the double layer capacitance (C_{dl}) were obtained. The data is reported as the inverse of R_{ct}, or admittance, in mhos cm^{-2}. The maximum phase angle between the imposed potential and induced current was determined from a Bode plot of the data.

Statistical analysis

The admittance from three WE electrodes in each experimental monoculture and mixed culture experiment were compared to the sterile control by the Student's *t*-test.

EXPERIMENTAL RESULTS

OCP and biofilm formation

The biofilm formation on the WE resulted in a >300 mV decrease in the OCP that was not seen in the sterile control (Fig. 2). In separate experiments, preliminary evidence based on AODC and PLFA determinations on coupons recovered at various times after inoculation indicated that the drop in OCP corresponded to the formation of a biofilm on the WE surface. The initial OCP was between −250 and −300 mV(SCE) for all kettles. The OCP, in the presence of D, decreased to −550 mV(SCE) after 90 h. In the presence of H, the OCP decreased to −600 mV(SCE) by 70 h. The OCP decreased to −650 mV(SCE) in 40 h for the D + H diculture. In the presence of B, H, and D, the OCP decreased at a constant rate from −300 to −610 mV(SCE) in 70 h.

Differential corrosion with microbial biofilms of different composition

The average corrosion rate, measured with EIS as admittance (mhos cm^{-2}) for the sterile control and the different monocultures and combinations with time is illustrated in Figs 3 and 4. All values represent the average of three measurements from three WE. Standard deviations are indicated at the 2 day or 4 day time points and at the end of the experiments. In Fig. 3 the change in admittance for the monocultures shows a rapid initial increase for all organisms compared to the control. All reach a maximum in the 2–4 day time period and then decrease so that by the end of the experiment (16 and 17 days) the monocultures all show a smaller admittance than the sterile control. No viable organisms were recovered from the D biofilm. The time course of corrosion measured as admittance with combinations of the organisms showed the same initial more rapid increase than the sterile control as the monocultures (Fig. 4). All the mixed cultures containing D showed a significantly higher admittance than the control or the monocultures after the first 4 days. Of

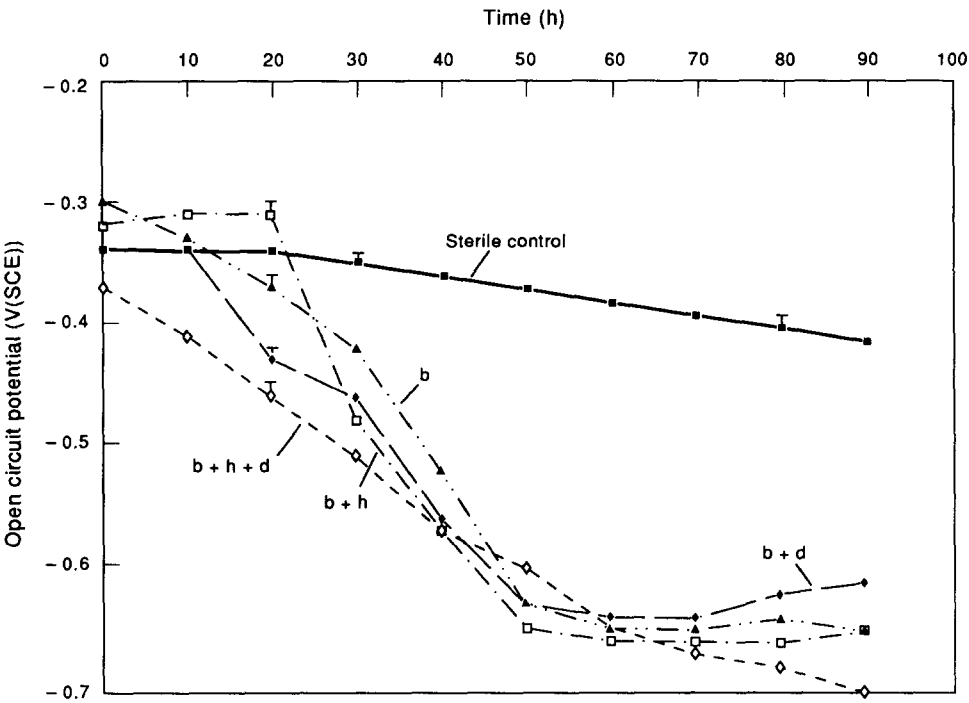

FIG. 2. Change in open circuit potential (OCP) with colonization of working electrode. *Bacillus* sp. (B), *Hafnia alvei* (H) and *Desulfovibrio gigas* (D) in combinations compared to the sterile control.

particular note is the rapid increase and sustained higher corrosion rate for the H + D diculture over all other combinations. The admittance for all combinations with D in consortia were statistically different from the control at $P < 0.01$ (Table 1).

The frequency of the maximum phase angle between the imposed potential and the induced current was at 8.5 Hz for the sterile control, 2.3 Hz for the B + H and 0.7 Hz for the triculture.

Recovery of organisms from the working electrode surfaces

At the end of the experiments illustrated in Figs 2–4, the glass cylinder was clamped on the electrode and the biofilm organisms recovered for analysis by viable counts and MPN or extraction and PFLA analysis. The three organisms utilized in this study had specific signature PLFA (Table 2). A signature PLFA is found in a high mol% in one but not the other of the three microbes. These can then be compared to the universal palmitic acid (16:0) which is found in all three organisms.

The proportions of the microbes actually recovered from the working electrodes at the end of the experiments of Figs 2–4 are listed in Table 3. The viable biomass based on the total viable and MPN counts and the total PLFA show reasonably good agreement (second and third columns). The proportions based on the viable and MPN counts are compared to the proportions estimated by the magnitude of the ratio of signature PLFA/16:0 in the microbes found at the end of the experiment (fourth and fifth columns). The two methods give divergent estimates for the

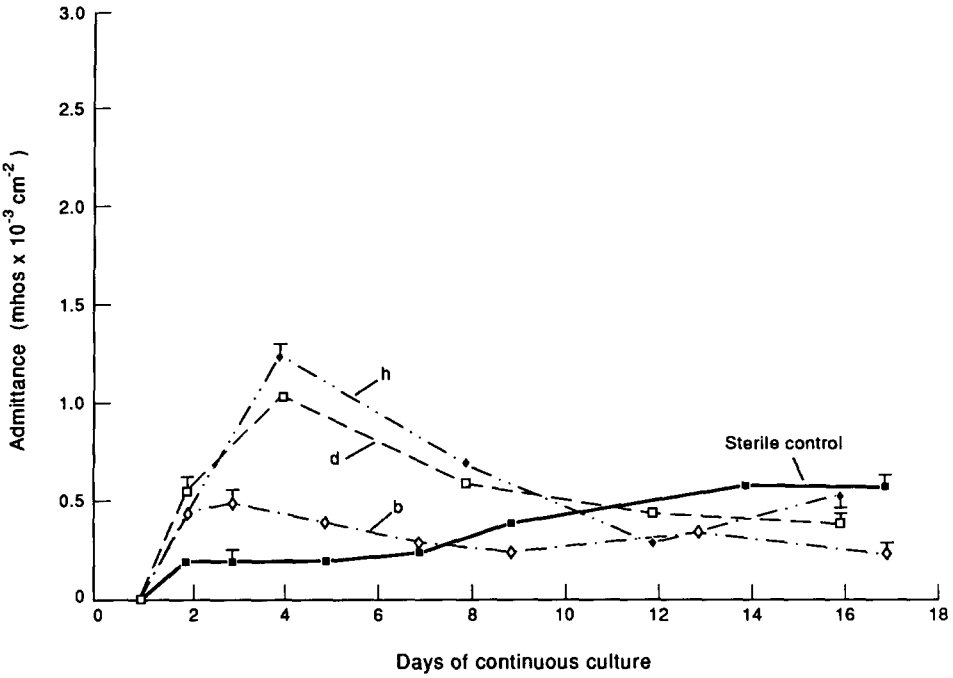

FIG. 3. Change in corrosion rate measure as admittance (R_v^{-1}, cm^{-2}) by EIS on mild steel coupons incubated in the flow-through aparatus plotted as mean ($n = 3$) with standard deviation indicated on the third (20 h) sample. Monocultures, combinations of bacteria, and sterile control indicated as in Fig. 2.

proportions of B + H with the viable counts indicating a higher proportion of B and the PLFA ratio indicating the H to be dominant. For the B + D and H + D communities the much higher proportions of aerobe to the SRB are found with both methods with the greatest disparity in the proportions of H + D. With the triculture the relative proportions agree with both methods. Another way to establish the relationships between the communities is to compare the similarities of the total PLFA pattern of each sample using cluster analysis (Fig. 5). The triculture and the H + D are dominated by the H pattern of straight chain PLFA. The B pattern of branched chain PLFA occurs only with the B monoculture and the B + D electrodes. The B + H pattern appears intermediate and none shows influence of the PLFA of D.

With the PLFA analysis it is possible to show that the growth on the electrodes in the continuous culture flow-cell with dilute bulk phase results in a changed physiological state (Table 4). Compared to the inoculum cultures the microbes isolated from the electrodes show a higher ratio of *trans/cis* 18 carbon monoenoic PLFA. With the exception of the high levels of cy17:0 in the H inoculum (possibly due to recovery in stationary growth phase), the organisms recovered from the electrodes show an accumulation of the cyclopropane PLFA.

Comparison with direct environmental enrichments

A corrosion tubercle was recovered from a 4 inch carbon steel pipe transmitting

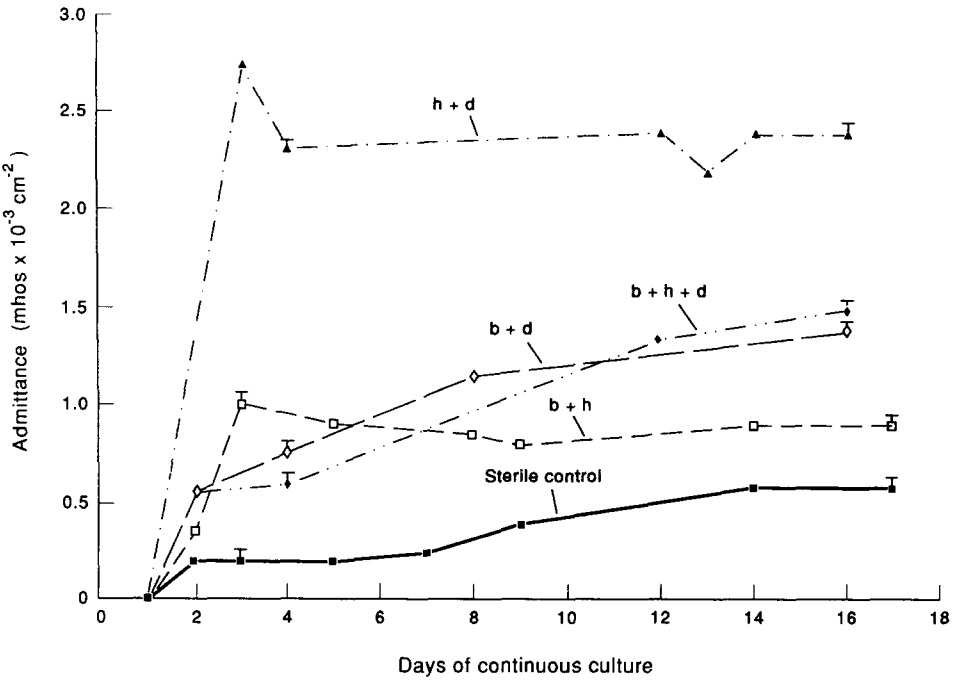

FIG. 4. Average corrosion rate measured as admittance (R_v^{-1}, cm^{-1}) by EIS on mild steel electrode coupons measured in the flow-through apparatus. Monocultures, combinations of bacteria, and sterile control are indicated as in Fig. 2. Data plotted as mean ($n = 3$) with standard deviation indicated to show 10–15% deviation of the mean.

TABLE 1. THE AVERAGE ADMITTANCE OF CARBON STEEL ASSOCIATED WITH *Bacillus* sp. (B), *Hafnia alvei* (H) AND *Desulfovibrio gigas* (D) IN MONOCULTURE AND IN CONSORTIA AFTER 15 DAYS (ISOLATES); AEROBIC (A), FERMENTATIVE (F) AND SRB (S) ENRICHMENTS MEASURED AFTER 17 DAYS

Bacterial community	Admittance* 10^{-3} ohms cm^{-2}	*t*-test significance from control
Isolates		
Control	0.53 ± 0.01	
B	0.53 ± 0.01	
H	0.86 ± 0.23	$P < 0.25$
B + H	0.74 ± 0.02	$P < 0.20$
D	0.72 ± 0.08	$P < 0.20$
B + D	1.40 ± 0.14	$P < 0.01$
H + D	2.30 ± 0.25	$P < 0.01$
B + H + D	1.40 ± 0.11	$P < 0.01$
Enrichments		
Control	0.6 ± 0.07	
A + F	1.4 ± 0.25	$P < 0.01$
A + F + S	3.1 ± 0.28	$P < 0.01$

*Average ± one standard deviation ($n = 3$).

TABLE 2. MOL% OF SIGNATURE PLFA RECOVERED FROM WORKING ELECTRODES*

	Organism mol%		
PLFA	B	H	D
i15:0	35	0	7
i17:1	6	0	23
cy17:0	0	37	0
16:0	4	22	37

*Mol% average ($n = 2$); organisms are designated as in Figs 1–3.

lake water and the microbiota characterized by PLFA. A portion was incubated with aeration (aerobic), a second with no aeration (fermentative), and the third anaerobically to recover lactate utilizing SRB (sulfate-reducing). The cluster analysis of PLFA patterns showed the original tubercle microbial community most closely approximated the SRB (Fig. 6). The corrosion rate after incubation for 17 days is given in Table 1. The ratio of viable counts of the heterotrophic bacteria to the MPN of the SRB was 10^5 to 1.

DISCUSSION

The flow-through glass kettles containing the multiple sided WE carbon steel electrodes allowed on-line, non-destructive determination of biofilm formation by different monocultures and combinations of isolates (Figs 1 and 2). The corrosion rate estimated by the admittance was readily determined in this system by EIS. The ±5 mV sinusoidal perturbations used in EIS have been shown not to affect the cell numbers, viability, and metabolic activity of the attached biofilm[12] and allow on-line

TABLE 3. MICROBES RECOVERED FROM WORKING ELECTRODES

Organisms	Biomass		Proportions					
			Viable count			PLFA estimate		
Inoculated	Viable count	PLFA estimate	B	H	T	B	H	T
D	N.D.	N.D.						0.6
H	4.3×10^7	1.8×10^7					1.6	
B	1.0×10^7	1.4×10^7				9.0		
B + H	1.1×10^8	6.0×10^7	100	1		0.3	0.9	
B + D	2.5×10^6	8.0×10^6	500		1	14.0		1.3
H + D	4.0×10^8	6.0×10^8		10^4	1		1.4	0.07
H + B + D	3.2×10^7	1.8×10^8	20	300	1	0.002	1.1	0.0?

Organisms, designated as in Table 1, were recycled at the end of the experiment. Biomass was estimated by viable counts (B, H.) and MPN (D) were contrasted to PLFA, calculated using 100×10^{-6} moles PLFA per 10^{12} cells g^{-1} dry weight. Proportions of B, H and T were estimated from viable counts and ratios of signature PLFAs to 16:0. Signature PLFAs were B—i15:0, H—cy19:0 and D—i17:1 (Table 2).

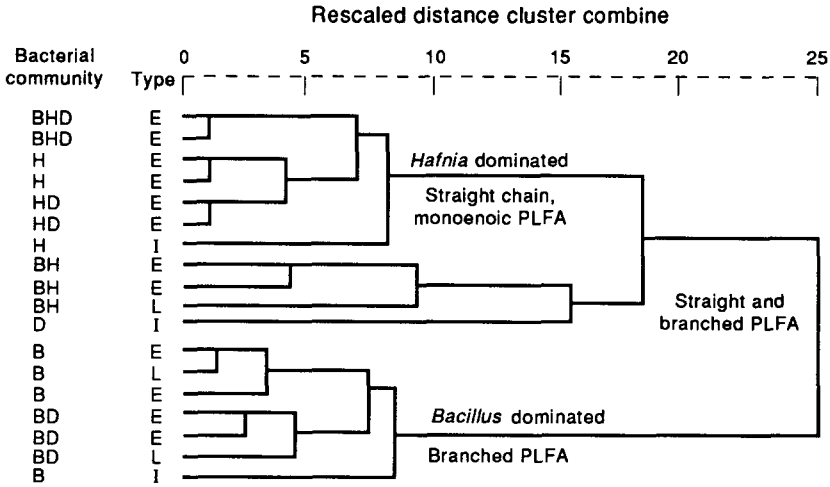

FIG. 5. Cluster analysis of bacterial combinations for correlation analysis based on relatedness of total pattern of PLFA. Bacteria and combinations of bacteria indicated as in Fig. 2. The type refers to organism recovered from the electrode surface (E), the bulk phase liquid from the continuous culture system (L) and the inocula (I).

measurements during the course of the experiments. EIS determination of R_v has been shown to be equivalent to the standard distructive DC linear polarization analysis.[13] The reproducibility of the EIS measurements could be determined by positioning each face of the four-sided electrode adjacent to the Luggin probe for EIS analysis.

With this system it was possible to show that the monocultures of each organism (including the non-viable D) formed a biofilm and initially induced greater corrosion than the sterile control. With time the corrosion decreased to that of the sterile control. Combinations of bacteria also accelerated the initial rates of corrosion and the subsequent corrosion did not fall to that of the control. The combinations which included D induced higher rates of corrosion and the specific combination of H + D induced the greatest corrosion (Figs 3 and 4, Table 1).

Examining the biomass and the composition of the biofilms on the WE at the end of the experiment confirmed that the evidence that different combinations of bacteria grown in the same bulk phase liquid on the same substratum show markedly different MIC activities. This was determined by the traditional viable counts (MPN

TABLE 4. INDICATIONS OF PHYSIOLOGICAL STATUS OF MICROBIOTA RECOVERED FROM ELECTRODES

Organisms PLFA Ratios	Inoculum		Electrode			
	H	D	H	H + D	H + B	H + B + D
18:1w7t/7c	0.04	0	0.26	0.30	0.22	0.14
cy17:0/16:1w7c	30.0	0	6.9	0.6	4.1	4.8
cy19:0/18:1w7c	0	0.1	0.9	0.6	0.7	0

Organisms are designated as in Figs 1–3.

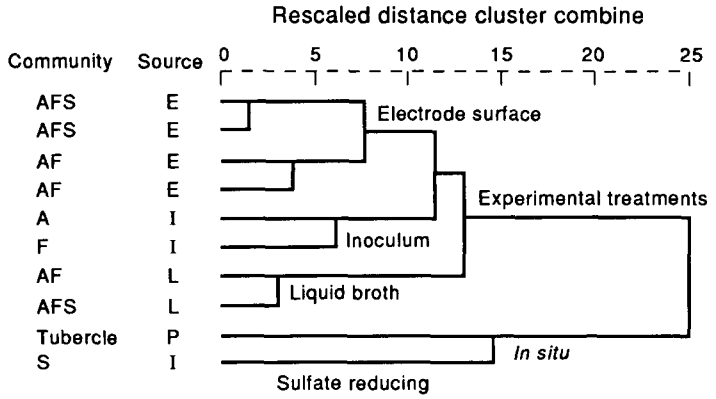

FIG. 6. Cluster analysis of enrichments from carbon steel corrosion tubercle used as innuocula for biofilm formation on the working electrodes in the continuous flow test system using patterns of PLFA. Community from aerobic (A), facultative (F), sulfate-reducing (S), and the tubercle (T). Source of the communities was the electrode (E), the inocula from the enrichments (I), the bulk phase of the culture apparatus (L) and the pipe (P).

for the SRB) and by the signature PLFA method which does not require that the organisms be quantitatively isolated from the substratum or that they be grown in culture. The three organisms utilized have distinctive signature PLFA patterns (Table 2). The total biomass recovered from the coupons ranged between 10^6 and 10^9 by viable count and from 10^6 to 10^8 by PLFA (Table 3). PLFA has been shown to be an accurate estimate of the viable microbial community.[14] The biomass of microbiota shows no consistent relationship with the corrosion rate. The composition of the biofilm communities could be examined by these techniques and was shown to markedly effect the corrosion rates. The estimates for the community composition based on ratios of signatures to 16:0 generally agreed with viable counts except for the B +H diculture where the PLFA indicated the H community dominated slightly compared to a 10^2 greater B based on viable counts. As expected the relationships based on cluster analysis of the total community PLFA patterns followed the estimates by the signature ratios (Table 3, Fig. 5). One general tendency resulting from the community analysis was that the greater the ratio of heterotrophic bacteria to SRB in the biofilm the greater the corrosion rate. The ratios varied from 10–100 heterotrophs to 1 SRB (B + D, triculture), 10^2–10^4 to 1 (H + D), and 10^5 to 1 in the tubercule enrichments.

Utilizing the direct PLFA analysis of the biofilm on the electrodes provided additional insights into the microbial community. The test system with flowing diluted bulk phase generated PLFA patterns associated with starvation and toxic stresses (increased monoenoic *trans/cis* ratios and increased cyclopropane to monoenoic PLFA ratios) (Table 4). These are conditions in which secondary metabolite and exopolymer formation are induced.[12] These metabolic states could accelerate MIC (Ford and Mitchell). Utilizing PLFA pattern analysis it was also possible to show that the community recovered from an actual fresh corrosion tubercle on a carbon steel pipe more closely resembled the PLFA pattern of the SRB than the heterotrophic bacteria (Fig. 6). The more complex communities generated directly from the enrichments (Fig. 6) which included the SRB produced the some 30% greater corrosion rate than the H + D diculture (Table 3).

This study clearly documents that the MIC corrosion rate measured under carefully controlled conditions with a biofilm on the same substratum of carbon steel formed in the same flowing dilute bulk phase is markedly affected by the microbial community structure. MIC is not directly related to the total biofilm biomass but is to specific combinations of microbes in that biofilm.

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