Effect of Hydrogenase and Mixed Sulfate-Reducing Bacterial Populations on the Corrosion of Steel

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The importance of hydrogenase activity to corrosion of steel was assessed by using mixed populations of sulfate-reducing bacteria isolated from corroded and noncorroded oil pipelines. Biofilms which developed on the steel studs contained detectable numbers of sulfate-reducing bacteria (10^4 increasing to $10^7/0.5\text{ cm}^2$). However, the biofilm with active hydrogenase activity (i.e., corrosion pipeline organisms), as measured by a semiquantitative commercial kit, was associated with a significantly higher corrosion rate (7.79 mm/year) relative to noncorrosive biofilm (0.48 mm/year) with 10^5 sulfate-reducing bacteria per 0.5 cm^2 but no measurable hydrogenase activity. The importance of hydrogenase and the microbial sulfate-reducing bacterial population making up the biofilm are discussed relative to biocorrosion.

The hydrogenase enzyme of the sulfate-reducing bacteria (SRB) may participate in the initiation of the corrosion process by removing the cathodic hydrogen from steel (4, 11, 13). However, much of the work related to hydrogenase involvement in metal corrosion has been done in laboratory studies, in which it has now been observed that, after repeated subculturing, SRB strains showed lower hydrogenase activity relative to newly isolated organisms (8). Accordingly, our laboratory has been studying field samples, and to date we have examined in excess of 150 samples from oil pipeline systems (secondary oil recovery water systems, corrosion coupons, and steel studs for biofilm development) for evidence of biocorrosion by comparing a new commercial hydrogenase test kit with the conventional culture media for SRB detection. The hydrogenase test is broad based and detects a number of sulfidogenic, hydrogenase-containing genera including the classical SRB such as *Desulfovibrio* spp., *Desulfomonas* spp., and *Desulfotomaculum* spp. and the nonclassical genera such as *Clostridium* spp. and *Shewanella* spp. (6). In 93% of the samples tested, there was a positive association between hydrogenase activity and the detection of SRB (6). Metal sections of pipe removed from the pitted areas showed significant hydrogenase activity (i.e., +++; see Materials and Methods for details). In a small percentage of cases (i.e., 4.5%), detectable numbers of SRB were present but no hydrogenase activity or corrosion was detected. These observations emphasize the difficulties encountered when enumeration of SRB only is used as an indicator of biocorrosion.

It was further observed that, when mixed cultures isolated from corroded areas of the pipeline originally testing positive for hydrogenase were subcultured into enriched Butlin's medium (see Materials and Methods), the hydrogenase activity then tested negative. Also, a mixed SRB culture isolated from a noncorroded pipe section had no detectable hydrogenase activity. Thus, hydrogenase activity present in the SRB may influence the biocorrosion process and also be subject to repression or induction control mechanisms. These two contrasting consortia of SRB presented an ideal system to compare the effects of different SRB populations,

and in particular the enzyme hydrogenase, on the corrosion of steel.

MATERIALS AND METHODS

Robbins device. Butlin's medium (5) was circulated at 25°C through two Robbins devices (7) at a flow rate of 4 liters/min. The Robbins device, a circular tube (15-mm diameter by 1 m) made of Admiralty brass, had cleaned and preweighed cylindrical steel studs (SAE 1020; $\text{Fe}^0 = 99.4\%$; 0.5-cm^2 surface area) inserted into sample ports which were separated from the brass casings by O rings. The device was connected to a 5-liter sterile reservoir, containing Butlin's (5) salts medium, by polyvinyl chloride tubing, and nitrogen gas was bubbled through the reservoir to facilitate the development of the anaerobic SRB population.

Growth conditions. The cultures used in this study were mixed populations of SRB isolated from corroded and noncorroded areas of pipelines as described above. Biofilm scrapings from the pipes were inoculated into enriched Butlin's medium (5) supplemented with 0.1% Casamino Acids and 1.0% tryptone; hydrogenase activity of the scrapings was analyzed by the Caproco hydrogenase test (see below for details), which was positive for the corroded scrapings and negative for the noncorroded scrapings. Within 72 h the media turned black, indicative of hydrogen sulfide production. However, both cultures gave a negative hydrogenase test when grown on the enriched medium. The mixed nature of the populations was verified by the diverse cellular sizes and morphology as observed by direct microscopic examination and by the diverse morphology on enriched Butlin's agar medium. The mixed populations of each sample type were batch cultured in 50 ml of Butlin's enriched medium at 37°C for 72 h and then added to the reservoir of separate Robbins devices. The devices were subsequently designated loop 1 and loop 2; loop 1 contained the organisms originally testing positive for hydrogenase activity, while loop 2 contained the organisms which originally gave a negative hydrogenase test. The carbon source, lactate, was added at a concentration of 10 g/liter, and the pH of Butlin's medium was adjusted to 7.0.

Analyses for SRB, hydrogenases, lactate and acetate, and metal dissolution. At periodic intervals, the reservoirs of the

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loops and randomly selected studs were examined for SRB counts and hydrogenase activity. Determinations of the amount of lactate and acetate present in the reservoir were also made. The SRB counts were determined by using the standard most-probable-number technique (7) in which 1-ml aliquots of the appropriate dilution tubes were used to inoculate triplicate culture tubes, each containing a sterile nail in Butlin's medium. The subsequent development of a black tube was indicative of SRB growth. To enumerate the SRB attached to the surface of the mild steel studs, the preweighed studs were first placed in 10 ml of 4% sodium citrate solution and cleaned ultrasonically (12). This procedure gently removed any biofilm bacteria present on the stud. Subsequently, 1 ml was used to perform the most-probable-number assay as described above, and the remaining 9 ml was used to perform a semiquantitative hydrogenase test as described in the Caproco 1987 instruction manual (Caproco Ltd., Edmonton, Alberta, Canada). The hydrogenase test provided a measure of the presence of the enzyme and a crude rating of enzyme activity as strong (+++), medium (++), or weak (+). The scoring system was a relative one and was based on the activity of a hydrogenase from a known sulfidogenic microorganism, with +++ representing 5 to 5,000 nmol of hydrogen uptake per min, ++ representing 0.05 to 5 nmol of hydrogen uptake per min, and + representing 0 to 0.5 nmol of hydrogen uptake per min. The hydrogenase test on the reservoir medium (planktonic samples) was conducted on a 9-ml subsample, as described above for the biofilm sample. Results from the hydrogenase test were obtained within 4 h at 37°C.

Lactate was measured by the UV/enzymatic test kit method of Boehringer Mannheim (1989) in which the reduction of NAD^+ to NADH in the conversion of lactate to pyruvate by lactate dehydrogenase was used as an indicator of quantity of lactate. Acetate was measured on a Perkin-Elmer Sigma 3B gas chromatograph equipped with a flame ionization detector and using a glass column (2 m by 2 mm) packed with 10% Fluorad FC-431 plus 1% H_3PO_4 on Chromosorb W-HP (80/100 mesh). The column temperature was 130°C, and the carrier gas, N_2 , flowed at a rate of 30 ml/min.

The metal studs were removed at different times during the course of the experiment. The biofilms were removed from the studs by placing them in 10 ml of 4% sodium citrate solution, and the studs were cleaned ultrasonically. The cleaned studs were dried and weighed; the difference from the initial weight was reported as weight loss in Tables 1 and 2. New studs (preweighed) replaced the ones removed from the Robbins device. As an additional control, clean studs were placed in sterile medium contained in anaerobic jars and analyzed for weight loss after 64 days.

At the conclusion of the experiment, a stud with a complete biofilm and a second stud with the biofilm removed were sampled from each loop and prepared for scanning electron microscopy (SEM) by the procedure of Bryant et al. (3). For purposes of comparison, a clean fresh stud was also prepared for SEM.

RESULTS AND DISCUSSION

Table 1 summarizes the results from the loop 1 experiment in which studs from the Robbins device were periodically removed and visually examined for the development of a biofilm. Since none was observed during the first 17 days, estimates of the numbers of SRB on the stud were not done. However, some studs were subjected to the Caproco hydrogenase test kit. No hydrogenase was detected on these

studs or from planktonic samples tested, in spite of the fact that a population of SRB was present and growing in the liquid in which the number of SRB at day 1 was $1.4 \times 10^3/\text{ml}$ and had risen to 10^7 by day 23. The appearance of the end product acetate was due to the initial metabolism of lactate, but after 8 days it was quickly metabolized at a rate similar to that of lactate by the SRB; such an observation indicated the mixed nature of the SRB population present (Fig. 1). Interestingly, the metal loss in the first 23 days was also minimal, and the metal loss measurements for this period were averaged to obtain an average metal loss for the first 23 days (Table 1).

After 23 days, the first biofilm on the steel studs which recorded a strong positive hydrogenase test (i.e., +++) was noted along with a measurable number of SRB (i.e., $1.4 \times 10^4/0.5 \text{ cm}^2$). Hydrogenase detection in the biofilm was probably caused by a shift from chemoorganic to chemolithotrophic metabolism by some of the organisms in the mixed biofilm population because of the very low level of utilizable carbon sources (lactate and acetate) remaining in the recycling medium (Fig. 1). This induction of hydrogenase activity was possibly due to those organisms being able to utilize cathodically produced hydrogen as an alternate energy source. In the recycling liquid, though, there were still 10^7 planktonic SRB per ml which for the first time gave a weak positive hydrogenase test. The constant level of planktonic SRB from day 23 onwards was likely due to the depletion of the carbon sources lactate and acetate (Fig. 1), and presumably the low planktonic hydrogenase test result was related to the sloughing off of hydrogenase-positive cells from the stud to the liquid phase (1). From days 23 to 49, the number of SRB detected on the studs increased from 10^4 to $10^6/0.5 \text{ cm}^2$, and all samples tested had a positive hydrogenase test. Taking the corrosion rate at day 23 as the new starting point for the period from days 23 to 49, it was

FIG. 1. Metabolism of lactate and acetate in loop 1.

TABLE 1. Hydrogenase activity, numbers of SRB, and weight loss measurements of studs from loop 1

Sample (days)	Hydrogenase activity ^a		No. of SRB		Stud wt loss (g)	Corrosion rate, avg (mm/yr) ^b
	Stud	Medium	Stud, per 0.5 cm ²	Medium, per ml		
1	—	—	ND ^c	1.4×10^3	ND	
8	—	—	ND	2.5×10^4	0.0093	
	—	—	ND		0.0116	
9	—	—	ND	ND	0.0046	
	—	—	ND		0.0132	
14	—	—	ND	ND	0.0121	
	—	—	ND		0.0180	
17	—	—	ND	ND	0.0099	
23	+++	+	1.14×10^4	1.1×10^7	0.0084	
Avg					0.0109 ± 0.0037^d	0.44 ± 0.10
28	+++	+++	ND	ND	0.0098	
35	++	++	ND	ND	0.0173	
37	ND	ND	ND	4.5×10^6	ND	
43	++	++	ND	2.5×10^6	ND	
49	+++	+++	3.8×10^6	ND	$0.0371 (n = 4)$	
Avg					0.0214 ± 0.0141	0.89 ± 0.38
57	+++	+	ND	2.5×10^6	0.1274	
64	+++	+	1.4×10^7	1.1×10^6	0.1084	
Avg					0.1179 ± 0.013	7.79 ± 0.86
Control studs after 64 days (avg)					$0.0009 (n = 2) \pm 0.0003$	0.01 ± 0.004

^a +++, strong; ++, medium; +, weak; —, none.^b Corrosion rate (millimeters per year) = MPY $\times$ 0.0254. MPY = $[534.6 \times 1,000 \times \text{weight loss (g)}]/[\text{density mild steel} \times \text{surface area (in}^2) \times \text{time (h)}]$.^c ND, not determined.^d Standard deviation of the mean.

observed that the corrosion rate more than doubled, going from 0.44 to 0.89 mm/year. The corrosion rate in the control studs in the anaerobic jars remained insignificantly low throughout the experiment at 0.01 mm/year.

With the hydrogenase-active bacteria now firmly established, the corrosion rate on the stud rose dramatically to 7.79 mm/year from days 49 to 64. This corrosion rate was likely a reflection not only of hydrogenase activity but also of the metabolic end products that the cells were making, such as H₂S, which reacted with the anodically produced metal ion Fe²⁺ to form the black corrosion product FeS, which was observed on these biofilms.

The data from the first 23 days of this study can be viewed as a control in which high numbers of detectable SRB were present in the liquid and yet no hydrogenase activity or no significant corrosion rate was detected. When the cell population switched its metabolism from using lactate and acetate as an energy source to using the metal (cathodically produced hydrogen) as an energy source, there was a concomitant increase in hydrogenase activity. The continued presence of the growing biofilm with a strong hydrogenase activity was associated with the dramatic increase in the corrosion rate. The SEM of the studs at 64 days shows the presence of a biofilm (Fig. 2B, C, and D), which, when removed, reveals the typical honeycomb metal dissolution pattern of mild steel (10, 14) that had taken place (Fig. 3B and D). Both pictures can be compared with the control stud in which even the concentric rings from the lathe cut are evident (Fig. 2A and 3A and C).

In the case of loop 2, detectable numbers of SRB were

cultured from both the metal stud and the liquid medium early in the growth phase (Table 2). The number increased over the first 18 days, which was in agreement with the consumption of 90% of the lactate and acetate in a pattern similar to that shown in Fig. 1. The early appearance of detectable SRB on the metal at day 4 was in sharp contrast to the situation in loop 1, in which no SRB were found on the metal while lactate was present in the reservoir. Presumably, this was a reflection of the different populations of SRB in the two loops. Although viable SRB counts decreased from 10⁵ to 10¹/ml in the liquid phase after 35 days, SRB numbers on the metal studs remained relatively constant throughout the experiment. At no time, however, was the hydrogenase activity detected, and minimal corrosion activity of the steel was evident on the basis of weight loss measurements. The corrosion rate was 0.14 mm/year during the first 29 days, and it increased slightly to 0.48 mm/year over the next 32 days. Quite clearly, loop 1 has an active corrosive biofilm relative to loop 2, the state of which was also indirectly indicated by the absence of hydrogenase activity in loop 2. The association of active hydrogenase activity with corrosion rate also agrees with another study in which strong hydrogenase activity (++++) correlated with corrosion rates of 6.35 mm/year (corrosometry measurement) while none was observed with negative hydrogenase tests (6). In other works, active hydrogenase was also associated with augmented corrosion rates (2, 4).

These loop experiments are consistent with some of the anomalies reported by researchers in the field who have recorded high numbers of SRB in some locations with no

FIG. 2. SEMs of steel studs from loop 1 showing the presence of a developed biofilm. (A) Control stud with concentric rings from lathe cut present; bar, 500 μm . (B) Biofilm deposit; bar, 500 μm . (C) Biofilm deposit with bacterial rods clearly visible; bar, 5 μm . (D) Biofilm deposit with presence of curved *Desulfovibrio*-type rods clearly visible; bar, 0.5 μm .

FIG. 3. SEMs of steel studs from loop 1 originally with biofilm as per Fig. 2 but now with biofilm removed. (A) Control stud with concentric rings from lathe cut present; bar, 500 μm . (B) Stud after biofilm removed; notice honeycomb pitting effect; bar, 500 μm . (C) Close-up view of control stud; bar, 5 μm ; (D) close-up view of steel stud with biofilm removed; bar, 5 μm .

TABLE 2. Hydrogenase activity, numbers of SRB, and weight loss measurements of studs from loop 2^a

Days	Hydrogenase activity		No. of SRB		Wt loss (g)	Corrosion rate, avg (mm/yr)
	Stud	Medium	Stud, per 0.5 cm ²	Medium, per ml		
0	—	—	ND ^b	2.5×10^5	0	
4	—	—	1.1×10^4	4.5×10^5	0.0014	
8	—	—	1.15×10^4		0.0054	
11	—	—	4.5×10^5		0.0027	
15	—	—	4.5×10^5		0.0052	
18	—	—	ND	9.5×10^5	0.0040	
21	—	—	ND	4.5×10^5	0.0031	
29	—	—	4.5×10^4		0.0092	
Avg					0.0044 ± 0.0025	0.14 ± 0.08
35	—	—	1.5×10^4	4.5×10^1	0.0164	
41	—	—	ND		0.0197	
54	—	—	1.5×10^4	2.5×10^1	0.0144	
61	—	—	ND		0.0160	
Avg					0.0166 ± 0.0022	0.48 ± 0.06

^a For details, see Table 1, footnotes a, b, and d.^b ND, not determined.

subsequent corrosion and in other places have correlated low numbers with high corrosion of the mild steel; the correlation of corrosion rate with numbers of SRB is questionable. Although the enzyme analysis for hydrogenase activity was semiquantitative, the presence of hydrogenase may be interpreted as a good indicator of biocorrosion activity. Clearly, the susceptibility of a system to biocorrosion is dependent on a number of variables, two of which have been suggested in this investigation, namely, the microbial makeup of the mixed population of SRB and the presence or absence of the enzyme hydrogenase.

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