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Axenic aerobic biofilms inhibit corrosion of SAE 1018 steel through oxygen depletion

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Abstract Corrosion inhibition of SAE 1018 steel by pure-culture biofilms of *Pseudomonas fragi* and *Escherichia coli* DH5α has been evaluated in complex Luria-Bertani medium, seawater-mimicking medium, and modified Baar's medium at 30 °C. In batch cultures, both bacteria inhibited corrosion three to six fold compared to sterile controls, and the corrosion was comparable to that observed in anaerobic sterile media. To corroborate this result, a continuous reactor and electrochemical impedance spectroscopy were used to show that both *P. fragi* K and *E. coli* DH5α decreased the corrosion rate by 4- to 40-fold as compared to sterile controls; this matched the decrease in corrosion found with sterile medium in the absence of oxygen and with *E. coli* DH5α grown anaerobically. In addition, the requirement for live respiring cells was demonstrated by the increase in the corrosion rate that was observed upon killing the *P. fragi* K biofilm in continuous cultures, and it was shown that fermentation products do not cause an increase in corrosion. Hence, pure-culture biofilms inhibit corrosion of SAE 1018 steel by depleting oxygen at the metal surface.

Introduction

Pseudomonads (and other aerobic bacteria) have been shown to both increase and decrease the rate of metal corrosion through polymer production during biofilm formation (Black et al. 1988; Nivens et al. 1986). Jack et al. (1992) found that monocultures of an aerobic *Bacillus* sp. induced greater corrosion of mild steel initially, but that the rate of this corrosion decreased to that of a sterile control after 17 days. Aerobic bacteria can also create localized anaerobic niches in a biofilm that allows sulfate-reducing bacteria to colonize and increase corrosion (Hamilton 1983). However, under certain favorable conditions, pseudomonads have also been able to prevent corrosion (Jayaraman et al. 1997; Pedersen and Hermansson 1989, 1991). Thomas et al. (1988) suggest the presence of a biofilm may act as a diffusional barrier to the corrosive substances and prevent them from reaching the metal surface.

The role of oxygen depletion by bacterial biofilms and its effect on corrosion inhibition has not been clearly delineated. Jayaraman et al. (1997) recently reported the lack of corrosion inhibition with a dead biofilm as compared to a live biofilm. Hernandez et al. (1994) also observed the need for live cells to decrease the corrosion rate and suggested that the biofilm may not act as a physical barrier to corrosive agents but, instead, that it reduces the concentration of oxygen at the metal surface by respiration and hence reduces corrosion. Pedersen and Hermansson (1991) reduced the oxygen concentration of sterile media to that observed in bacterial suspensions, but were unable to observe any corresponding decrease in corrosion.

To elucidate the role of oxygen with protective biofilms, electrochemical impedance spectroscopy (EIS) is useful. EIS is a specialized corrosion-monitoring technique that has been used in laboratory studies of microbially induced corrosion and also in monitoring corrosion in the field (Mansfeld 1995). Using EIS, the open-circuit potential (E_{oc}, or the corrosion potential, E_{corr}) and the polarization resistance (R_p) may be measured. Although the E_{oc} is a simple parameter to measure and gives a reliable indication of biofilm formation, it provides little information about the mechanism of corrosion (Mansfeld 1995). The Stern-Geary equation (Stern and Geary 1957) gives the correlation between the corrosion current, i_{corr}, and R_p as

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$$i_{corr} = \frac{\beta_A \beta_C}{2.303(R_p)(\beta_A + \beta_C)}$$

where β_A and β_C are the anodic and cathodic Tafel slopes respectively. Hence, R_p is inversely proportional to the corrosion rate (Stern 1958; Stern and Roth 1957; Macdonald and McKubre 1987). Using EIS, instantaneous and accurate relative corrosion rates can be determined without disturbing the biofilm, which eliminate the need to use mass loss to calculate corrosion rates.

The ability of bacteria to form regenerative biofilms offers a cost-efficient alternative to conventional methods of corrosion prevention like organic coatings (Jayaraman et al. 1997), provided that such a biofilm can also restrict the colonization of sulfate-reducing bacteria. Failure of materials because of biofilm formation is not associated solely with the chemical process industry; biofilm formation and loss of integrity of catheters and implants is a common problem observed with medical devices (Dall et al. 1990; Gorman et al. 1993; Mellonig et al. 1995). Mellonig et al. (1995) also report a loss of osseous support material, which was partly due to bacterial growth on the implant. Hence it seems reasonable to contemplate harnessing the protective ability of biofilms to reduce the chances of failure of medical implants and to reduce corrosion in industrial settings. Though the results presented in this study are of axenic cultures for aerobic bacteria (which are unlikely to be encountered in natural environments although some of these experiments were conducted in seawater-mimicking medium to simulate a marine environment), they are useful for studying the corrosion mechanisms taking place at a colonized metal surface. These data can then be used to develop strategies for combating systems where sulfate-reducing bacteria colonize an aerobic biofilm and cause localized corrosion.

In this study, the growth of *Pseudomonas fragi* K and *Escherichia coli* DH5α(pKMY319) was examined and its effect on corrosion inhibition of SAE 1018 steel in batch and continuous cultures recorded. Anaerobically grown *E. coli* DH5α and anaerobic sterile controls were used to obtain continuous corrosion rates in the absence of oxygen, using EIS measurements, and to understand the mechanism of corrosion inhibition by pure-culture biofilms.

Materials and methods

Bacterial strains, growth media, culture conditions, and specific growth rates

A kanamycin-resistant, transposon mutant of the spoiled-meat bacterium, *P. fragi* ATCC 4973 (*P. fragi* K; Jayaraman et al. 1997), and a tetracycline-resistant enteric bacterium, *E. coli* DH5α(pKMY319) (Jayaraman et al. 1997) were used on the basis of their reported ability to form biofilms (Huang et al. 1994; Parolis et al. 1991) and inhibit corrosion (Jayaraman et al. 1997). *P. fragi* K and *E. coli* DH5α(pKMY319) were streaked separately from −85 °C glycerol stocks onto Luria-Bertani (LB) medium/agar plates with 50 μg/ml kanamycin and 25 μg/ml tetracycline respectively. A single colony of each was then picked, used to inoculate 10 ml growth medium, and grown overnight at 30 °C and 250 rpm (series 25 shaker; New Brunswick Scientific, Edison, N.J.). Biofilms were developed in LB medium (Maniatis et al. 1982), Vätäänen nine-salts solution (VNSS) (Hernandez et al. 1994), and modified Baar's medium (American Type Culture Collection medium 1249) using batch and continuous cultures containing either tetracycline or kanamycin. The specific growth rate (Bailey and Ollis 1986) was determined by monitoring cell growth using a Spectronic 20D spectrophotometer as reported previously (Jayaraman et al. 1997).

Metal coupon preparation and determination of mass loss

SAE 1018 steel coupons (for batch shake-flask experiments, weighing 4.9–5.1 g and having a diameter of 25.5 mm and a thickness of 1.2 mm) or plates (for continuous-culture experiments, 7.5-cm squares with a thickness of 1.2 mm) were cut from sheet stock and polished with 240-grit polishing paper (Buehler, Lake Bluff, Ill.) as reported previously (Jayaraman et al. 1997). The specific mass loss observed in batch cultures (in mg/cm^2, determined by dividing the total weight loss by the total surface area of the coupon, 11.18 cm^2, after scrubbing the coupon with a rubber stopper) was used as an indicator of the extent of corrosion.

Batch- and continuous-culture corrosion experiments

Batch corrosion experiments were conducted with three replicates of SAE 1018 steel coupons in QorPak bottles (60 ml volume; Fisher Scientific Co., Pittsburgh, Pa.) with foam stoppers under static conditions. Twelve ml sample of medium was used in all experiments, and the metal coupons were placed at the bottom of the Qorpak bottles. For batch-culture anaerobic experiments, the QorPak bottles were placed inside a GasPak chamber (Fisher Scientific Co, Pittsburgh, Pa.) and a fully anaerobic environment was generated and maintained (as shown by a methylene blue anaerobic indicator strip; Fisher Scientific Co, Pittsburgh, Pa.). A 0.1% (v/v) inoculum from a late-exponential-phase culture was used for all batch- and continuous-culture experiments.

A continuous reactor was designed and constructed for monitoring corrosion rates with EIS in flow systems (Fig. 1). The metal sample formed the bottom of the reactor, the four corners of the metal sample were not part of the reactor, a glass cylinder (5.5 cm diameter, 0.6 cm thick) formed the walls of the system, and a 1-cm-thick Teflon plate (12.6 cm × 12.6 cm) formed the roof of the reactor. The working volume of the reactor was 100 ml with an airflow rate of 200 ml/min (FM1050 series flow meter; Matheson Gas Company, Cucamonga, Calif.). An anaerobic environment was generated in the continuous reactors by adding oxygen-free nitrogen (99.998%, Liquid Carbonic, Oak Brook, Ill.) initially to the headspace above the liquid at 200 ml/min for 1 h then reducing the flow to 25 ml/min. The growth temperature was maintained at 30 °C by heating tape wrapped around the reactor. Sterile controls were operated at a nutrient flow rate of 12 ml/h using a Masterflex precision standard drive with a ten-turn potentiometer (Cole-Parmer, Niles, Ill.). Biofilms were allowed to develop for 12 h in batch mode then nutrients were added continuously, and biofilm development was monitored using EIS. The dilution rate (nutrient flow rate divided by reactor volume) used in the reactors with bacteria depended on the growth rate of the bacterium (Jayaraman et al. 1997) and was typically 30%–50% of the specific growth rate (μ_{max}, Bailey and Ollis 1986). Whenever the flow rate of the medium was varied, at least four reactor volumes of liquid (400 ml) were passed before the system was considered to be at steady state. The sample metal specimen was at the bottom of the reactor with a titanium counter electrode at the centre (3.8 cm in diameter, positioned 1.5 cm above the metal plate) and an autoclavable reference electrode at the periphery (3.0 cm above the metal plate).

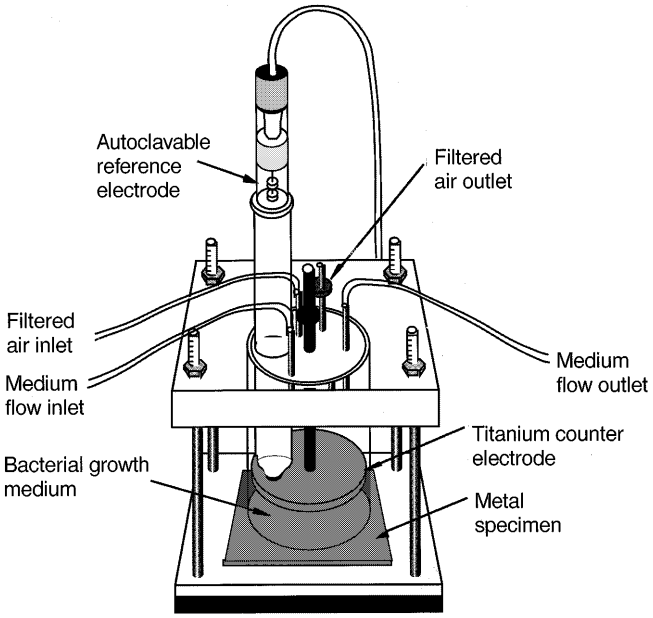

Fig. 1 Schematic diagram of the continuous reactor designed for the electrochemical impedance spectroscopy measurements (100 ml working volume)

Electrochemical impedance spectroscopy (EIS)

Polarization resistance and open-circuit potential data were obtained from a.c. impedance data using a Solarton-Schlumberger electrochemical measurement unit (SI 1280; Schlumberger Technical Instruments Division, San Jose, Calif.) interfaced to a Macintosh computer (PowerMac 7100/80; Apple Computers, Cupertino, Calif.) running EISIS electrochemical experimentation software (University of California, Irvine). The titanium electrode was used as the counter electrode and measurements were made over a frequency range of 20000–0.001 Hz at fixed time intervals. The reference electrode was an Ingold Silver Scavenger DPAS electrode (model 105053334; Mettler-Toledo Process Analytical Inc., Wilmington, Mass.). The polarization resistance was determined as the low-frequency value of the impedance (where the imaginary part of the impedance was zero or negligible), and E_{oc} was measured as the potential between the metal specimen and the counter electrode (relative to the reference electrode).

Results

The results presented below are from uncontaminated pure cultures (confirmed by periodic plating of a reactor sample on LB/agar plates) of antibiotic-resistant *P. fragi* (Jayaraman et al. 1997) and *E. coli* DH5α, which were used in all experiments to avoid the contamination seen previously in long-term EIS experiments (Hernandez et al. 1994).

Corrosion inhibition with aerobic and anaerobic batch cultures

Mass loss from the mild-steel coupons in LB medium, VNSS medium, modified Baar's medium, and distilled water with *P. fragi* K and *E. coli* DH5α(pKMY319) was examined after 14 days in stationary batch cultures at 30 °C under either aerobic or anaerobic conditions (Fig. 2). The least mass loss was observed with anaerobic sterile media and anaerobically grown *E. coli* DH5α(pKMY319), which had nearly identical results: 10- to 20-fold lower mass loss than that observed in aerobic sterile media. This shows conclusively that fermentation products are not responsible for this corrosion. Furthermore, since corrosion of SAE 1018 steel under anaerobic conditions was reproducibly inhibited 10-fold in three different kinds of bacterial growth media, the corrosion inhibition was not due to selective media constituents, which agrees with the observations of Pedersen and Hermansson (1989). Similar mass loss trends were observed with metal coupons in distilled water; the corrosion was nearly 10-fold less under an-

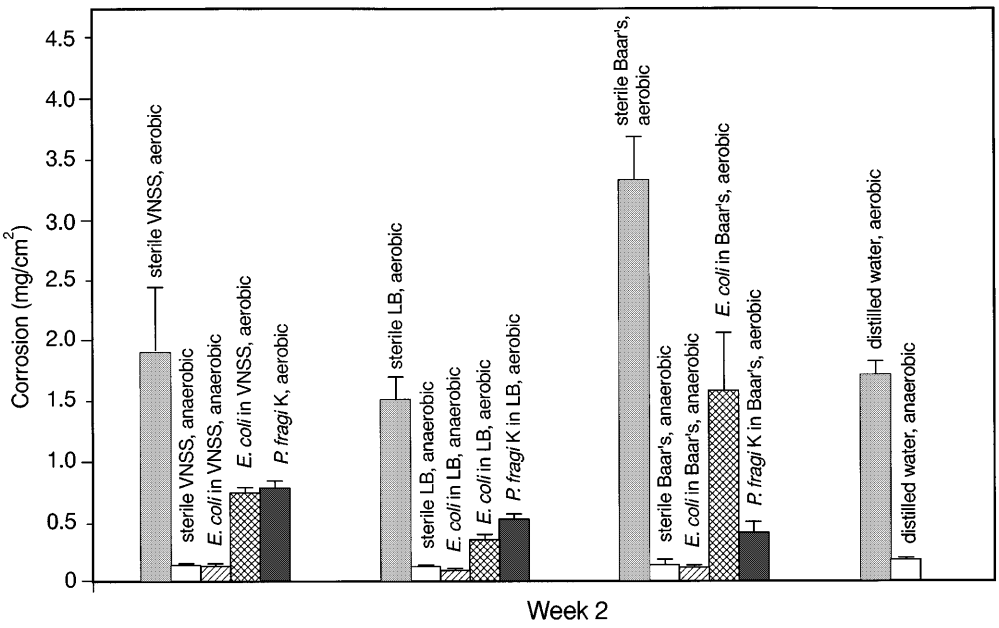

Fig. 2 Batch corrosion data ($n = 3$) to interpret the role of oxygen depletion in corrosion inhibition using sterile controls, *Pseudomonas fragi* K, and aerobic and anaerobically grown *Escherichia coli* in different growth media at 30 °C (standard deviation errors bars shown). *VNSS* Vätäänen nine-salts solution, *LB* Luria-Bertani medium, *Baar*'s modified Baar's medium

aerobic conditions. No difference in mass loss was observed between anaerobic LB sterile controls generated by Gaspak (0.125 mg/cm^2) and those flushed with nitrogen (0.131 mg/cm^2), hence the hydrogen generated by the Gaspak did not affect corrosion of the mild-steel coupons.

Metal coupons immersed in aerobic bacterial suspensions showed a 3- to 7-fold decrease in mass loss compared to aerobic sterile controls. The mass loss observed with *P. fragi* K did not vary significantly with the growth medium used, and corrosion inhibition (as a percentage of mass loss in the sterile controls) with this strain was least in VNSS medium. However, the mass loss observed with aerobically grown *E. coli* DH5α (pKMY319) varied with the growth medium; maximum corrosion inhibition was observed in rich LB medium, and the minimum corrosion inhibition occurred in modified Baar's medium.

Biofilm formation in continuous cultures

A decrease in the open-circuit potential (E_{oc}) was used as the indicator of biofilm formation on SAE 1018 steel surfaces. As shown in Fig. 3, both *P. fragi* K and *E. coli* DH5α(pKMY319) decreased E_{oc} of the SAE 1018 steel plate by 150–200 mV in LB medium and VNSS medium compared to sterile controls. The initial E_{oc} in LB medium and VNSS medium (before inoculation) was between 650 mV and 700 mV for all samples. Typically, E_{oc} decreased to 600 mV within 4–6 h after addition of the bacteria and decreased more slowly beyond 24 h. The E_{oc} observed with anaerobic sterile medium was lower than that observed with aerobic sterile medium (Fig. 3); a

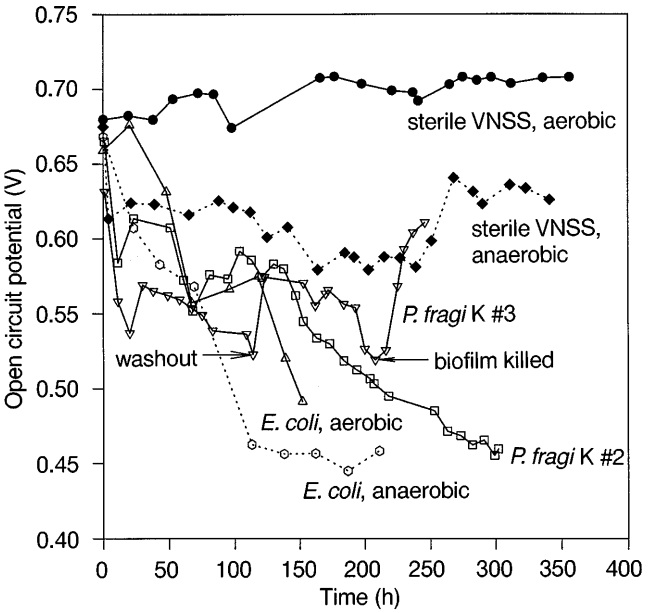

Fig. 3 Open-circuit potential, indicative of biofilm formation in continuous reactors, from electrochemical impedence spectroscopy experiments using sterile controls, *P. fragi* K, and *E. coli* in VNSS medium at 30 °C. Data shown are from representative runs (all runs summarized in Table 1)

Table 1 Biofilm formation and corrosion inhibition of SAE 1018 steel in different bacterial growth media under aerobic and anaerobic conditions. The open-circuit potential, E_{oc}, and R_p values are steady-state values obtained at the end of the experiment. *VNSS* vätäänen nine-salts solution

Medium	O_2 /N_2	Bacterium	Duration, (h)	E_{oc}, (mV)	log (*R*p), Ω	Specific growth rate, (h^{-1})
Luria-Bertani	O_2	Sterile	340	690–700	2.7	–
	O_2	Sterile	340	680–700	2.8	–
	O_2	*P. fragi* K	350	490–500	3.3	0.64 ± 0.02[a]
	O_2	*P. fragi* K	250	470–480	3.1 (live)	
			300	480–490	2.7 (dead)	
			370	600–610	2.1 (dead)	
VNSS	O_2	Sterile	510	690–700	2.3	–
	N_2	Sterile	340	610–620	2.9	–
	N_2	Sterile	330	620–630	2.8	–
	O_2	*P. fragi* K	110	530–540	3.3	0.36
	O_2	*P. fragi* K	325	460–470	3.8	
	O_2	*P. fragi* K	195	520–530	3.7 (live)	
			250	610–620	2.7 (dead)	
	O_2	*E. coli* DH5α(pKMY319)	150	490–500	3.1	0.28
	N_2	*E. coli* DH5α(pKMY319)	210	450–460	3.2	ND[b]
Modified Baar's	O_2	Sterile	300	690–700	2.2	–
	O_2	Sterile	370	690–700	2.1	–
	N_2	Sterile	310	570–580	3.0	–
	N_2	Sterile	250	600–610	2.9	–
	O_2	*P. fragi* K	310	500–510	3.4	0.37

[a] Data from Jayaraman et al. (1997)
[b] Not determined

decrease of 75–100 mV was observed over a period of 14 days. However, the decrease in E_{oc} observed with anaerobic sterile medium (60–100 mV) was never comparable to that observed in the presence of aerobically grown bacteria (200–250 mV). A similar decrease in E_{oc} was also observed with anaerobic sterile Baar's medium (Table 1). Anaerobically grown *E. coli* DH5α (pKMY319) in VNSS medium also decreased the E_{oc} of the reactors and was comparable to that observed with aerobically grown *E. coli* DH5α(pKMY319) (Fig. 3).

Corrosion rates with aerobic and anaerobic continuous cultures

Corrosion rates in LB medium and VNSS medium with *P. fragi* K and *E. coli* DH5α(pKMY319) at 30 °C were examined for 2 weeks (Fig. 4 and Table 1). Since the polarization resistance, $R_{p,}$ is inversely proportional to the rate of corrosion (Stern 1958; Stern and Roth 1957; Macdonald and McKubre 1987), changes in the polarization resistance were correlated to the extent of corrosion observed under various conditions. Irrespective of the type of growth medium and the presence or absence of bacteria, all reactors had an initial R_p value of 400–700 Ω. However, R_p for aerobic sterile controls in VNSS medium decreased to around 100–150 Ω within 12 h while R_p of *P. fragi* K and *E. coli* DH5α(pKMY319) in VNSS medium varied from 600 Ω to 6500 Ω. Therefore, by the Stern-Geary equation, the rate of corrosion was reduced 4- to 43-fold in VNSS medium. Aerobically grown *P. fragi* K also decreased the corrosion rate by 4- to 20-fold in LB medium and by 20-fold in modified Baar's medium (Table 1).

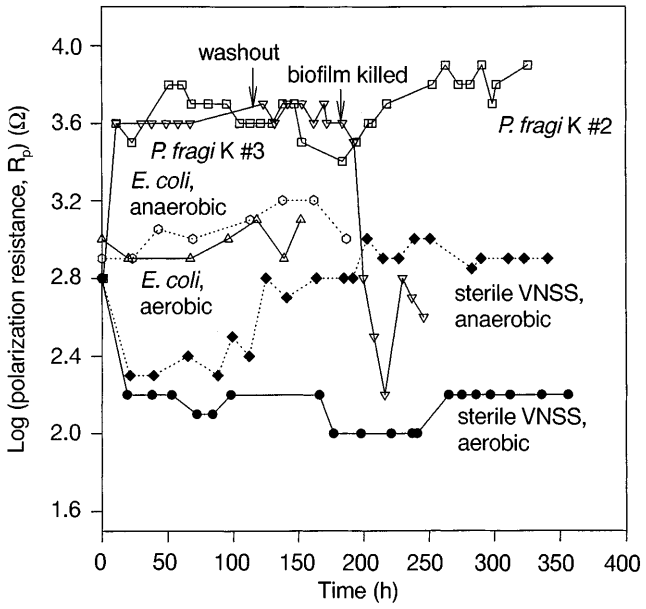

Fig. 4 Aerobic and anaerobic continuous polarization resistance values (R_p, which is inversely related to the corrosion rate) from electrochemical impedance spectroscopy experiments using sterile controls, *P. fragi* K, and *E. coli* in VNSS medium at 30 °C. Data shown are from representative runs (all runs summarized in Table 1)

Corrosion rates with anaerobic sterile medium and anaerobically grown *E. coli* DH5α(pKMY319) were also lower than that in aerobic sterile medium; however, the corrosion rates with anaerobic sterile medium were always greater than that observed in the presence of aerobically or anaerobically grown bacteria (Fig. 4). It was also observed that the corrosion rate in anaerobic sterile medium did not change significantly for 100 h (as shown by constant polarization resistance), beyond which there was a significant decrease in the corrosion rate (Fig. 4). However, the E_{oc} of the anaerobic sterile medium decreased by 100 mV within 12 h and remained relatively constant beyond 12 h (Fig. 3).

Effects of flow rate and dead cells on corrosion inhibition

Corrosion rates in the presence of *P. fragi* K in VNSS medium at 30 °C for 150 h at liquid medium flow rates of 11 ml/h and 72 ml/h (which correspond to 30% and 200% of the $\mu_{max} = 0.36\,h^{-1}$) and in LB medium at 30 °C for 14 days at various liquid medium flow rates of 19, 32, 45 and 128 ml/h (which correspond to 30%, 50% 70% and 200% of the $\mu_{max} = 0.6\ h^{-1}$) were obtained using EIS. No change in corrosion inhibition (R_p) was observed with varying flow rate. At each flow rate, the corrosion rate was always an order of magnitude (Fig. 4, 120–175 h) or a factor of 3–6 (not shown) less than rates in aerobic sterile controls. Nor did changes in the medium flow rate affect the E_{oc} significantly; E_{oc} of sterile aerobic media remained constant and nearly 150 mV greater than that observed in the presence of bacteria (Fig. 3).

To determine the role of a live biofilm in corrosion inhibition, a *P. fragi* K biofilm was developed in VNSS medium for 8 days in a continuous reactor; the biofilm was then killed by the continuous addition of 200 μg/ml chloramphenicol (185–250 h, Fig. 4) to the reactor, and the corrosion rate increased by one order of magnitude after 48 h of exposure to the antibiotic. A similar increase in corrosion rate by a factor of three after 48 h of exposure to the antibiotic was also observed in a continuous reactor when a *P. fragi* K biofilm in LB medium was killed by the continuous addition of 200 μg/ml chloramphenicol; the corrosion rate increased further by nearly one order of magnitude after 120 h of exposure (Table 1). This concentration of chloramphenicol was chosen since earlier shake-flask studies had shown that *P. fragi* K in suspension cultures was completely killed by 25 μg/ml chloramphenicol; however, sessile bacteria exhibit an increased resistance to antibiotics (Hoyle et al. 1990; Suci et al. 1994). Batch experiments in our laboratory have also shown that *P. fragi* K biofilms are killed by 200 μg/ml chloramphenicol, as shown by the lack of growth of a treated biofilm sample on LB/agar plates (some growth was obtained along the main streak path after 48 h).

Discussion

In this study, batch cultures of *P. fragi* K and aerobically and anaerobically grown *E. coli* DH5α(pKMY319) inhibited corrosion of SAE 1018 steel in LB medium, VNSS medium, and modified Baar's medium and reduced corrosion to levels comparable to those in sterile anaerobic media (Fig. 2). These results suggest the respiring biofilm cells decrease corrosion by depleting oxygen and differ from those of Pedersen and Hermannson (1991), who reported that lowering the oxygen content in sterile medium to 25% of that observed in a sterile control did not inhibit corrosion of ASTM A619 steel.

A continuous reactor (Fig. 1) was designed to permit non-destructive, real-time data acquisition and determination of biofilm formation and corrosion rates. The E_{oc} of the reactors with bacterial suspensions decreased by nearly 200 mV after 8–10 days of cultivation. A comparable decrease in E_{oc} of 100–200 mV in the presence of biofilm-forming bacteria has been reported by Jack et al. (1992) and Hernandez et al. (1994). The E_{oc} of sterile aerobic media (LB, VNSS, and modified Baar's medium) remained virtually unchanged for the duration of the experiment. In contrast, it was observed that the addition of bacteria to the reactor caused a decrease in E_{oc} in 3–6 h, and it has been reported that bacteria are capable of forming biofilms in this interval (Costerton et al. 1995). Our observations with confocal scanning laser microscopy confirm that *P. fragi* K forms a more robust and thicker biofilm than *E. coli* DH5α(pKMY319) (not shown); this agrees well with a slightly larger drop in E_{oc} for *P. fragi* K relative to *E. coli* DH5α(pKMY319) (200 mV compared to 150 mV, Table 1). Bacteria capable of forming thicker and more robust biofilms (e.g., *P. fluorescens*) caused a decrease of nearly 500 mV in E_{oc} under the same experimental conditions (not shown). A decrease of 75–100 mV in E_{oc} was also observed with anaerobic sterile medium. The E_{oc} value in the sterile reactors decreased by 75 mV rapidly during the first 12 h of exposure, remained relatively constant for almost 14 days, and did not decrease in a manner comparable to that observed with bacterial suspensions. Therefore, we propose that the 150- to 200-mV decrease in E_{oc} is due to formation of a biofilm.

The relative corrosion rate (as the inverse of the polarization resistance) in continuous reactors was also determined using EIS. *P. fragi* K and *E. coli* DH5α (pKMY319) biofilms led to a 10-fold decrease in corrosion relative to aerobic sterile medium. The high initial value of R_p observed in all experiments can be attributed to the time taken for the system to attain steady state. Hernandez et al. (1994) observed a decrease in corrosion of mild steel with pure cultures of *Pseudomonas* sp. S9 and *Serratia marscens*. However, Jack et al. (1992) have reported that pure cultures of a *Bacillus* species and *Hafnia alvei* formed a biofilm (decreased E_{oc} compared to sterile controls) and induced greater corrosion than sterile controls initially; but, the corrosion rate decreased to that of sterile controls after 17 days. In our experiments, bacterial suspensions always decreased the corrosion rate, which did not approach that of the sterile control after a similar exposure period (Fig. 4). This also agrees with our earlier observations (Jayaraman et al. 1997) in which there was a 2- to 5-fold inhibition of corrosion with different aerobic bacterial species in batch cultures over 4 weeks of exposure. In addition, our batch corrosion rates with bacteria were always less than those in the sterile controls. The continuous corrosion rates with anaerobic sterile medium were also lower than those of the aerobic sterile controls (Fig. 4).

This laboratory has also observed the lack of corrosion inhibition with cell-free, spent bacterial medium and with a dead biofilm (killed in situ) in batch cultures (Jayaraman et al. 1997). A similar experiment, using the continuous reactor and EIS, was performed here, in which a *P. fragi* K biofilm was killed and the system with the dead biofilm showed an increase in corrosion rate and E_{oc} within 48 h of antibiotic addition (Fig. 4 and Table 1). This clearly demonstrates the need for a live and actively respiring bacterial biofilm in corrosion inhibition, and the limited role for the biofilm as a passive barrier to diffusion of corrosive agents. Hernandez et al. (1994) also suggest the need for live bacterial cells, as they observed lower corrosion rates in the presence of live bacteria and higher corrosion rates with glutaraldehyde-killed cells (although there was no intact biofilm in their experiments).

The reactor system used for EIS testing had minimal mixing effects upon nutrient addition (fresh media added dropwise to quiescent liquid above the biofilm) because of the need for maintaining an intact, protective pure-culture biofilm on the metal surface and for minimizing the interference from turbulent flow in the EIS readings. Therefore, it is essential to partition the role of planktonic cells in the bulk supernatant and the attached biofilm in the observed corrosion inhibition. No significant difference in the corrosion rate was observed between a flow rate of 19 ml/h (corresponding to 30% of the maximum growth rate of the bacterium, μ_{max} and a turbid supernatant) and 128 ml/h (twice μ_{max} or washout in which no bacteria were present in the supernatant). Therefore, the number of cells in the supernatant (and hence the oxygen content of the supernatant) did not determine the degree of corrosion inhibition; the effects observed were due to the biofilm only and the changes in E_{oc} and R_p reported here were due to changes in the biofilm and not to suspended cells. This also helps in understanding our previous observations (Jayaraman et al. 1997) where a non-biofilm-forming *S. lividans* (as demonstrated by confocal scanning laser microscopy) did not inhibit corrosion in batch cultures; it was not possible to attribute the lack of corrosion inhibition to either the low turbidity (and increased presence of oxygen throughout the supernatant) or the absence of a uniform biofilm. However, the present observations clearly demonstrate the need for a live, respiring biofilm in corrosion inhibition.

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Note added in proof

To corroborate the a.c impedance corrosion rate data, the d.c. polarization technique (Macdonald and McKubre 1987) was used to calculate the corrosion rate of SAE 1018 mild steel from the cathodic and anodic Tafel slopes (β_A and β_c) and the Butler-Volmer equation (Raistrick 1987). Using this method, the polarization resistance (R_p) for mild steel in sterile modified Baar's medium was 155 ohms (corrosion rate of 1.97 milli inches per year) whereas the R_p with *P. fragi* K was 1950 ohms (corrosion rate of 0.19 milli inches per year). These R_p values are in excellent agreement with those obtained by the a.c. impedance technique (Table 1, cf. 142 ohms and 2512 ohms). The ratio of the corrosion rate in sterile medium and with *P. fragi* K is 10.4 which is also comparable to the ratio of 17.8 obtained with the steady-state R_p values obtained with the a.c. impedance technique (Table 1). Hence, both the a.c. and d.c. technique indicate that *P. fragi* K decreased the corrosion rate by an order of magnitude.

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