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Importance of biofilm formation for corrosion inhibition of SAE 1018 steel by axenic aerobic biofilms

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To investigate if corrosion inhibition by aerobic biofilms is a general phenomenon, carbon steel (SAE 1018) coupons were exposed to a complex liquid medium (Luria–Bertani) and seawater-mimicking medium (VNSS) containing fifteen different pure-culture bacterial suspensions representing seven genera. Compared to sterile controls, the mass loss in the presence of these bacteria (which are capable of developing a biofilm to various degrees) decreased by 2- to 15-fold. The extent of corrosion inhibition in LB medium depended on the nature of the biofilm: an increased proportion of live cells, observed with confocal scanning laser microscopy (CSLM) and image analysis, decreased corrosion. Corrosion inhibition in LB medium was greatest with *Pseudomonas putida* (good biofilm formation), while metal coupons exposed to *Streptomyces lividans* in LB medium (poor biofilm formation) corroded in a manner similar to the sterile controls. *Pseudomonas mendocina* KR1 reduced corrosion the most in VNSS. It appears that only a small layer of active, respiring cells is required to inhibit corrosion, and the corrosion inhibition observed is due to the attached biofilm.

Keywords: biofilms; corrosion inhibition; confocal scanning laser microscopy

Introduction

Biofilms are adherent microbial populations enclosed in a glycocalyx matrix [7]. Biofilms can develop on metal surfaces in natural environments and have been thought to increase the rate of corrosion [5,11,21]. *Pseudomonas* sp 200 increases corrosion of AISI 10–18 mild steel with increasing growth rate [24], and Jack *et al* [16] found that pure cultures of a *Bacillus* sp and *Hafnia alvei* induced greater corrosion of mild steel initially by 2- to 6-fold, but the rate of this corrosion decreased to that of a sterile control after 17 days. Corrosion occurs as a consequence of non-uniform biofilm formation and microcolony development on the metal surface which lead to the formation of oxygen concentration gradients and differential aeration cells near the metal surface [11].

However, aerobic bacteria (eg, pseudomonads, *Bacillus* strains) have been shown to decrease metal corrosion. Pedersen and Hermansson [25,26] reported 10-fold corrosion inhibition of SIS 1146 steel with cultures of *Pseudomonas* S9 and *Serratia marscens* EF190 after 19 days of exposure. Jayaraman *et al* [18] also observed a 2- to 10-fold reduction in corrosion of mild steel in LB medium in the presence of *Pseudomonas fragi* and *Escherichia coli* DH5α(pKMY319) over a period of 4 weeks. Therefore, aerobic biofilms can *decrease* corrosion compared to sterile controls in axenic cultures. These protective aerobic biofilms may ultimately enhance corrosion due to colonization of anaerobic pockets by sulphate-reducing bacteria (SRB) which cause an increase in corrosion [9,13,14,16] by the removal of hydrogen and also by the production of hydrogen sulphide and iron sulphide [20].

Conventional methods of corrosion prevention like the application of paints are expensive and require frequent maintenance [18]. Thus, the ability of bacteria to form biofilms which regenerate rapidly provides a cost-efficient alternative to the use of paints in preventing corrosion, provided such a biofilm can also restrict the colonization of SRB. Failure of materials due to biofilm formation is not only a problem associated with the chemical process industry. Biofilm formation and loss of integrity of catheters and implants is a common problem observed with medical devices [8,12,23]. Mellonig *et al* [23] report a loss of osseous support material which was partly due to bacterial growth on the implant. It may be feasible to utilize the protective ability of biofilms to reduce the chances of failure of medical implants.

The main objective of this study was to investigate the general nature of corrosion inhibition by protective biofilms by comparing corrosion of mild steel in batch cultures with fifteen species of aerobic bacteria in two different media: complex LB medium and the seawater-mimicking VNSS medium. A majority of the fifteen strains (*Bacillus*, pseudomonads, and *Escherichia* species) were chosen for their slime-forming ability [5] and are important in corrosion studies. The least and most protective biofilms in LB medium were visualized using confocal scanning laser microscopy and quantified using image analysis to determine the relative proportions of live cells, dead cells, and void to understand the differences in observed corrosion inhibition.

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Materials and methods

Bacterial strains, medium, and growth conditions

The fifteen different bacterial species are listed in Table 1. All of the strains were grown with metal coupons in 35 ml of Luria–Bertani (LB) medium [22] or Vatanen nine salts solution (VNSS) medium [15] supplemented with suitable antibiotics (Table 1) in 250-ml Erlenmeyer flasks without shaking at 30°C for 7 days. The sterile controls contained 100 μg ml^{-1} of ampicillin.

Metal coupon preparation and mass loss determination

SAE 1018 steel coupons weighing 4.9–5.1 g, and having a thickness of 1.2 mm were cut from sheet stock and polished with 240-grit polishing paper (Buehler, Lake Bluff, IL, USA). The polished samples were rinsed with distilled water, weighed (0.1 mg accuracy), cleaned ultrasonically (Fisher Scientific Co, Pittsburgh, PA, FS-3 series) in ethanol for 1 min, degreased with acetone, air-dried, and stored under vacuum. Samples (one coupon per flask) were autoclaved at 121°C for 20 min under a gravity cycle in dry Erlenmeyer flasks before the addition of growth medium and inoculation. All cultures were generated from stocks stored at –85°C in 20% (vol/vol) glycerol.

The mass loss observed (mg cm^{-2}) was used as an indicator of the extent of corrosion. After one week, each coupon was carefully removed from the growth medium. The biofilm was removed by repeated washing with distilled water and wiping the surface. The coupons were immediately dried and cleaned by scrubbing them with synthetic rubber under warm running water. All experiments were performed in triplicate.

Confocal scanning laser microscopy (CSLM)

Metal coupons with attached surface biofilms were removed carefully from Erlenmeyer flasks and immersed once in 3.0 ml of 0.85% NaCl to remove bulk supernatant cells. Cells were stained for 30 min by immersing the coupon in 3 ml of stain (1.5 μl of each stain component per ml) using the Live/Dead *Bac*lit bacteria viability assay kit (Molecular Probes, Eugene, OR, USA) and visualized with a MRC 1024 confocal microscope (Bio-Rad, Hercules, CA, USA). The images were analyzed using the COMOS software available on the MRC 600 confocal microscope [18]. Thin optical sections (0.5–1.0 μm) were collected over the complete biofilm thickness for a representative position (chosen as one of four similar positions in the biofilm on the same coupon).

Results

Mass loss with the various bacteria in LB medium and seawater-mimicking VNSS medium at 30°C was examined after 7 days of quiescent, batch culture (Table 1). The results show that the coupons submerged in the bacterial suspensions demonstrated a 2- (eg, *S. lividans*) to 15-fold (eg, *P. fluorescens, P. putida* KT2440 and *P. putida* F1) decrease in mass loss compared to the coupons immersed in sterile LB medium. A similar 2- (eg, *B. migulanus*) to 10-fold (eg, *P. mendocina* KR1) reduction in mass loss was observed in VNSS medium. The extent of corrosion inhibition varied markedly between the two media for some bacteria (eg, *P. putida* F1, *P. putida* KT2440, *S. lividans* TK23); however, 10 strains protected the metal notably well in both media (3- to 15-fold reduction in mass loss). Since *S. lividans* did not grow to high cell density in LB medium, but did grow well in VNSS medium (which contains 0.05% starch [34]), the higher cell density could have been responsible for the increased corrosion inhibition observed. No difference in corrosion was found with sterile controls in the presence or absence of 100 μg ml^{-1} of ampicillin (0.94 mg cm^{-2} in LB + amp *vs* 0.99 mg cm^{-2} in LB); hence, the antibiotics did not affect corrosion.

CSLM was used to visualize the biofilms developed in LB medium and discern a relationship between biofilm thickness, proportion of live and dead cells, and corrosion

Table 1 Bacterial strains, antibiotic resistances, and corrosion (mg cm^{-2}) of SAE 1018 steel coupons after 1 week in LB medium or VNSS medium with fifteen aerobic bacteria at 30°C. Data represent the average of three coupons and standard deviations are shown

Bacterium	Corrosion in LB (mg cm^{-2})	Corrosion in VNSS (mg cm^{-2})	Antibiotic resistance (μg ml^{-1})	Source [Ref.]
Sterile LB medium	1.03 ± 0.14	1.37 ± 0.15	–	–
Streptomyces lividans TK23.1	0.51 ± 0.08	0.24 ± 0.03	thiostrepton (50)	D Crawford [29]
Bacillus subtilis	0.39 ± 0.06	did not grow	–	ATCC 15134
Bacillus circulans	0.26 ± 0.06	0.37 ± 0.05	–	ATCC 31228
Rhizobium meliloti 102F34	0.18 ± 0.06	0.17 ± 0.02		M Sadowski [3]
Pseudomonas fragi K	0.17 ± 0.02	0.52 ± 0.08	kanamycin (100)	this lab [18]
Escherichia coli BK6	0.16 ± 0.04	0.45 ± 0.08	tetracycline (25)	this lab [31]
Bacillus brevis	0.14 ± 0.04	0.19 ± 0.07	–	ATCC 35690
Burkholderia cepacia G4	0.13 ± 0.04	0.22 ± 0.02	ampicillin (50)	M Shields [27]
Agrobacterium tumefaciens A114	0.13 ± 0.03	0.23 ± 0.05	–	EW Nester [30]
Bacillus migulanus	0.11 ± 0.02	0.74 ± 0.07	–	ATCC 9999
Escherichia coli HB101/pRK2013	0.11 ± 0.02	0.41 ± 0.05	kanamycin (50)	RW Frazee [10]
Pseudomonas mendocina KR1	0.10 ± 0.01	0.14 ± 0.02	ampicillin (50)	KM Yen [33]
Pseudomonas fluorescens 2-79	0.09 ± 0.01	0.36 ± 0.11	ampicillin (50)	USDA NRRL B15132
Pseudomonas putida KT2440	0.09 ± 0.02	0.38 ± 0.05	ampicillin (50)	M Bagdasarian [1]
Pseudomonas putida F1	0.07 ± 0.01	0.46 ± 0.02	ampicillin (50)	D Gibson [28]

inhibition. Bacterial biofilms were stained for live and dead cells and observed under the confocal microscope (Figure 1). The *Bac*lit stain distinguishes between live and dead cells based on cell membrane integrity; live cells have an intact membrane and stain green while dead cells do not have an intact membrane and stain red (Figure 1). The black space between the cell clusters in Figure 1 is the void space (not stained).

For the two strains that protected metal the most in LB medium, *P. putida* F1 and *P. putida* KT2440, robust biofilms were observed that developed to a thickness of approximately 15 μm and 12 μm, respectively, after 7 days of growth. The least-protective strains in LB medium, the *B. subtilis* biofilms, also developed to 15 μm (where present) after 7 days whereas *S. lividans* biofilms in LB medium were only 7 μm thick after a similar exposure period.

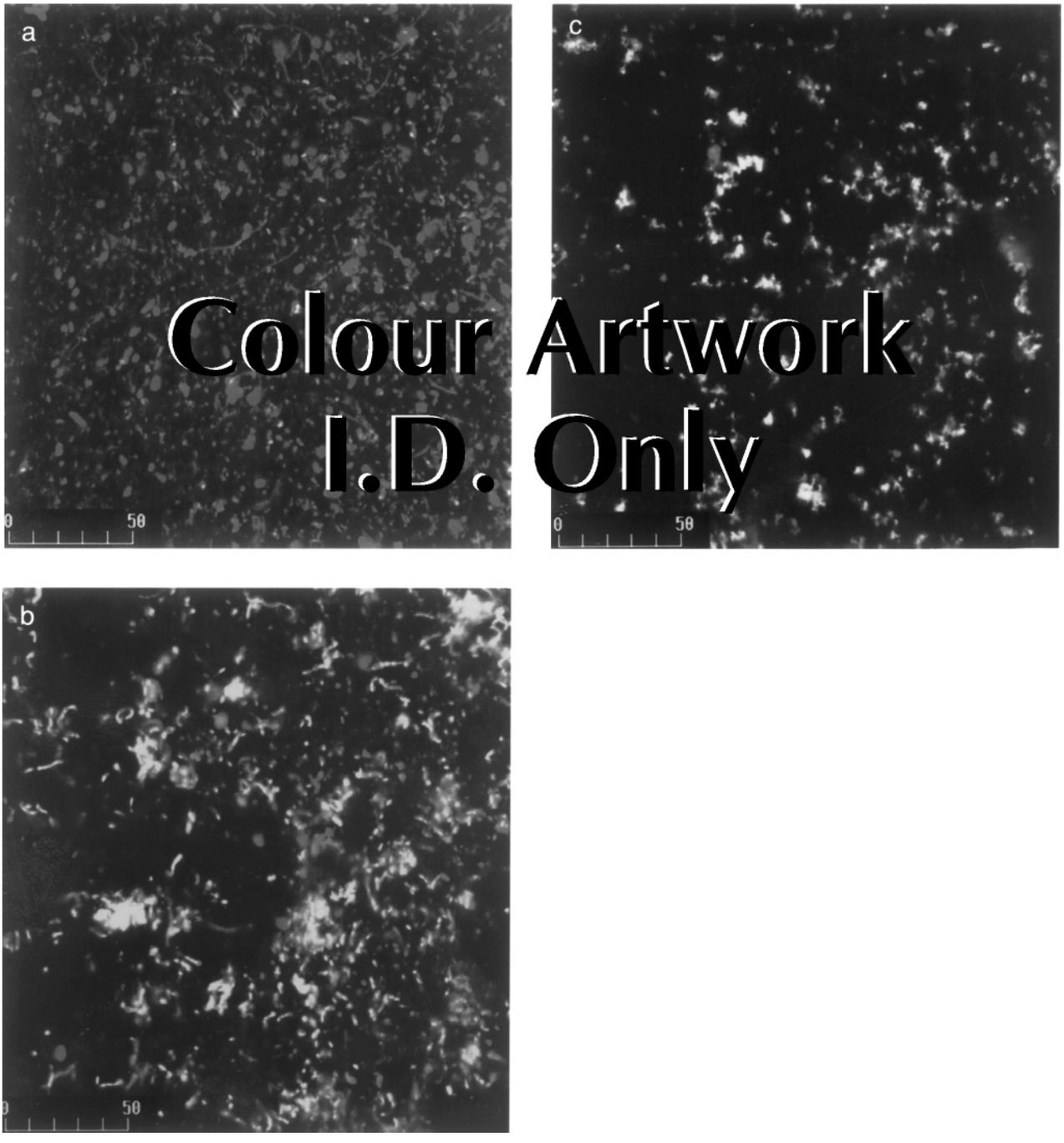

Figure 1 Confocal scanning microscope images of 7-day batch culture biofilms on SAE 1018 steel. (a) *P. putida* F1 in LB medium at 30°C stained for live (green) and dead (red) cells (image at a normalized depth of 0.7). (b) *B. subtilis* in LB medium at 30°C stained for live and dead cells (image at a normalized depth of 0.60). (c) *S. lividans* TK23.1 in LB medium at 30°C stained for live and dead cells (image at a normalized depth of 0.7). Scale bar represents 50 μm. Normalized depth is the position at which the image was obtained divided by the total biofilm thickness where position 0.0 is the biofilm–liquid interface and position 1.0 is the biofilm–metal interface.

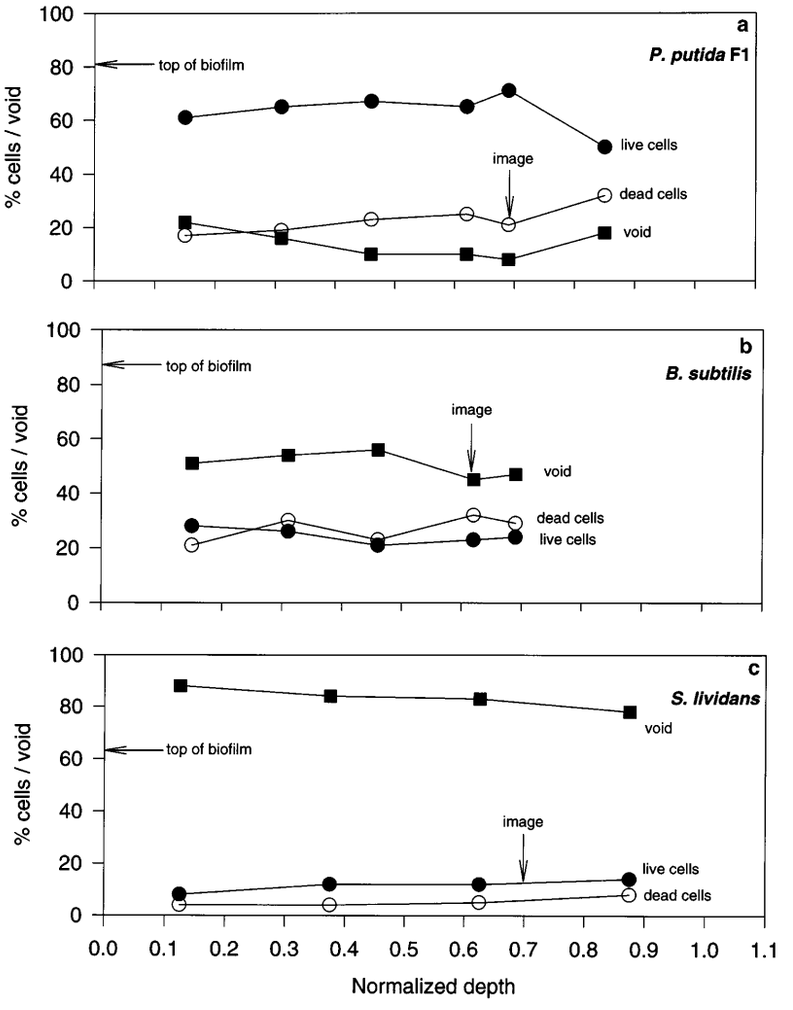

Figure 2 Depth profiles of 7-day batch culture *P. fragi* K biofilms developed on SAE 1018 steel in LB medium at 30°C. (a) *P. putida* F1; (b) *B. subtilis*; (c) *S. lividans* TK23.1. Measurements are of a representative position in the biofilm. Arrow indicates position where images in Figure 1 were obtained.

In addition, the relative proportions of live and dead cells varied significantly between the protective and less-protective strains in LB medium. The pseudomonad biofilms were packed densely with live cells (Figure 1a); for example, the *P. putida* F1 biofilm covered the metal coupon entirely and had nearly 70% live cells, 20% dead cells, and 10% void space at all depths of the biofilm (Figure 2a). A similar trend was also observed with *P. putida* KT2440 with nearly 50% live cells observed throughout the biofilm (not shown). In contrast, the *Bacillus* biofilm did not cover the metal surface uniformly since nearly 30% of the metal coupon did not have a biofilm or had only a very thin (~4 μm) biofilm. Where the biofilm was present, nearly equal proportions of live and dead cells were observed with a maximum of 23% live cells, 32% dead cells, and 45% void space at a representative depth of 3 μm from the coupon surface (Figure 2b). The *Streptomyces* biofilm also did not cover the metal coupon completely and, where the biofilm was present, it had approximately 15% live and dead cells throughout (Figures 1c and 2c).

The presence of *P. fragi* K and *E. coli* biofilms developed in VNSS medium on SAE 1018 coupons has also been verified [19] and can be considered as sufficient evidence for biofilm formation in VNSS medium by the other strains used in this study. Biofilms in VNSS medium differed from those in LB medium by the presence of copious amounts of exopolysaccharide (EPS), as compared to minimal EPS in LB medium, and less live and dead cells present at all depths in the biofilm [19].

Discussion

Assuming that the biofilm has approximately 10^9 cells ml^{-1} [6] in a uniform thickness of 15 μm which completely covers the metal surface and ignoring oxygen consumption by cells in the supernatant, the oxygen transfer rate can be calculated from the one-dimensional Fick's diffusion equation [4] to be approximately 2.7×10^{-10} mol O_2 s^{-1}. The amount of oxygen consumed by the biofilm is calculated from $\mu X/Y_{O2}$ [2] as 1.3×10^{-10} mol O_2 s^{-1}, where X is the biofilm cell density (g cells ml^{-1}), μ is the growth rate of the bacterium (0.65 h^{-1} for *P. fragi* K grown on LB medium

at 30°C [18]), and Y_{O2} is the yield factor relating the amount of cell mass (g) formed per g of oxygen consumed (0.85 for *P. fluorescens* grown on glucose [2]). Therefore nearly 50% of the maximum amount of oxygen which was transferred to the metal surface by diffusion was utilized by the bacteria in the biofilm. In addition, cells in the supernatant phase do consume oxygen and further reduce the amount of oxygen diffusing to the metal surface and hence available for corrosion. Even in the presence of a thick biofilm, oxygen can still probably diffuse to the metal surface through water channels [7] present in the biofilm. This could explain the relatively small amount of corrosion observed even in the presence of metabolically-active biofilms.

It should also be noted that the presence of a thick, aerobic biofilm on metal surfaces can result in the formation of localized anaerobic zones near the biofilm-metal interface [7]. The objective of the current study was to study the effects of pure culture aerobic biofilms on reducing generalized corrosion of carbon steel and hence should not be extrapolated to situations where a consortium of aerobic and anaerobic bacteria are present. Our laboratory is currently focusing on engineering biofilm-forming aerobic bacteria to exclude SRB from the biofilm to reduce the occurrence of localized corrosion.

The observed corrosion inhibition is due to the attached cells in the biofilm and not to oxygen consumption by the planktonic bacteria [17]. To show this, previous studies in our laboratory were performed with *P. fragi* K [18] at various liquid medium flow rates using electrochemical impedance spectroscopy to see the effect of planktonic cells on corrosion inhibition [17]. Corrosion rates were the same at flow rates of 19 and 42 ml h^{-1} (corresponding to 30% and 70% of the maximum growth rate of the bacterium, μ_{max} [18], and very turbid planktonic growth) and at 120 ml h^{-1} (twice μ_{max} or washout with no planktonic growth in the reactor). Hence, the presence of planktonic cells did not affect the polarization resistance which is inversely related to the corrosion rate by the Stern–Geary approximation [32]. These results agree well with the current observations that the efficiency of corrosion inhibition is influenced by the presence of a biofilm, and the extent of corrosion inhibition depends on the nature and robustness of the biofilm formed.

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