Appl Microbiol Biotechnol (2004) 65: 747–753
DOI 10.1007/s00253-004-1651-1

Rongjun Zuo · Thomas K. Wood

Inhibiting mild steel corrosion from sulfate-reducing and iron-oxidizing bacteria using gramicidin-S-producing biofilms

Received: 29 October 2003 / Revised: 8 April 2004 / Accepted: 23 April 2004 / Published online: 23 July 2004

Abstract A gramicidin-S-producing *Bacillus brevis* 18-3 biofilm was shown to reduce corrosion rates of mild steel by inhibiting both the sulfate-reducing bacterium *Desulfosporosinus orientis* and the iron-oxidizing bacterium *Leptothrix discophora* SP-6. When *L. discophora* SP-6 was introduced along with *D. orientis* to a non-antimicrobial-producing biofilm control, *Paenibacillus polymyxa* ATCC 10401, a corrosive synergy was created and mild steel coupons underwent more severe corrosion than when only *D. orientis* was present, showing a 2.3-fold increase via electrochemical impedance spectroscopy (EIS) and a 1.8-fold difference via mass-loss measurements. However, when a gramicidin-S-producing, protective *B. brevis* 18-3 biofilm was established on mild steel, the metal coupons were protected against the simultaneous attack of *D. orientis* and *L. discophora* SP-6. EIS data showed that the protective *B. brevis* 18-3 biofilm decreased the corrosion rate about 20-fold compared with the non-gramicidin-producing *P. polymyxa* ATCC 10401 biofilm control. The mass loss for the protected mild steel coupons was also significantly lower than that for the unprotected ones (4-fold decrease). Scanning electron microscope images corroborated the corrosion inhibition by the gramicidin-S-producing *B. brevis* biofilm on mild steel by showing that the metal surface remained untarnished, i.e., the polishing grooves were still visible after exposure to the simultaneous attack of the sulfate-reducing bacterium and the iron-oxidizing bacterium.

Introduction

Bacteria corrode metals (Borenstein 1994) and sulfate-reducing bacteria (SRB) are responsible for the corrosion of cast iron, carbon steel, stainless steel, and some alloys (Licina 1988). SRB corrosion damage in the United States results in losses of U.S. $ 4–6 billion/year (Beloglazov et al. 1991). Hydrogenase in SRB can utilize hydrogen as an electron donor to obtain energy and thus remove molecular hydrogen from the cathode, leading to cathodic depolarization of the metal surface (Geesey 1990). The corrosion product, iron sulfide, itself may deposit and become a cathode with a large surface area relative to the unreacted iron, accelerating the dissolution of the iron (Hamilton 1990). Lee et al. (1995) observed that the increase in iron sulfide concentration was accompanied by an increase in the corrosion rate of mild steel, which implicates SRB with corrosion. Furthermore, Booth et al. (1966) found that *Desulfosporosinus orientis*, a Gram-positive SRB, showed hydrogenase activity and caused corrosion damage of mild steel similar to that of other SRB. Tiller and Booth (1968) also found that the growth of *D. orientis* produced iron sulfide precipitation that stimulated the corrosion of aluminum.

The iron-oxidizing bacterium *Leptothrix discophora* SP-6 (Emerson and Ghiorse 1992) is a Gram-negative, sheath-forming, aerobic heterotroph growing at aerobic–anaerobic interfaces (Adams and Ghiorse 1986; Emerson and Ghiorse 1992; Brouwers et al. 2000). This bacterium is often found growing at the periphery of a sulfate-reducing area containing black iron sulfide and may cause a blockage in water distribution systems (Emerson and Ghiorse 1992). In addition, it was also discovered that manganese oxide deposited by *L. discophora* may initiate pitting corrosion of stainless steel (Olesen et al. 2001; Geiser et al. 2002) and may act as a cathodic reactant, increasing the corrosion of the metal on which it is deposited (Olesen et al. 2000).

Bacteria in biofilms dominate most ecosystems (Donlan and Costerton 2002; Dunne 2002). To combat the problems caused by some biofilms, biocide treatment is widely used (Fontana 1986; Franklin et al. 1991), but the results are frequently unsatisfactory (Hamilton 1985) since slowly-growing bacteria within biofilms are much less sensitive to antibiotic activity (Donlan and Costerton

R. Zuo · T. K. Wood (✉)
Departments of Chemical Engineering and Molecular and Cell Biology, University of Connecticut,
Storrs, CT 06269-3222, USA
e-mail: twood@engr.uconn.edu
Tel.: +1-860-486-2483
Fax: +1-860-486-2959

2002; Dunne 2002). High concentrations of biocides are also very expensive and cause environmental problems and even corrosion (Cord-Ruwisch et al. 1987; Franklin et al. 1991). Since microbial biofilms cause problems in both medicine (e.g., infections in human organs and biomedical devices; Costerton 2001; Gottenbos et al. 2002) and in industry (e.g., impeded heat flow over heat exchanger surfaces in food processing facilities, corrosion of concrete in sewage treatment plants; Kumar and Anand 1998; Maeda et al. 1999), it would be beneficial to control biofilms.

Gramicidin-S (Lee and Hodges 2003) is a 10-residue, cyclic peptide with a cyclo(-Val-Orn-Leu-D-Phe-Pro-)$_2$ structure containing the D-enantiomer of phenylalanine (D-Phe) and ornithine (Orn). Gramicidin-S is a very potent antimicrobial peptide isolated from soil, effective at killing a broad range of bacteria and fungi (Lee and Hodges 2003). Although the antimicrobial action of this peptide is not clear, it is believed that it interacts with the cell membrane, resulting in rupture and leakage of the cells (Wu and Hancock 1999; McInnes et al. 2000; Lee and Hodges 2003). Azuma et al. (1992) isolated two gramicidin-S-hyperproducing strains of *Bacillus brevis*, which secrete gramicidin-S at concentrations of 350 μg/ml (for *B. brevis* Nagano, a wild-type strain) and 590 μg/ml (for *B. brevis* 18-3, a chemical mutant of *B. brevis* Nagano). *B. brevis* 18-3 was used in this work.

Beneficial biofilms can decrease corrosion. Potekhina et al. (1999) and Jayaraman et al. (1997a, b, c) reported that the primary mechanism of corrosion reduction was the removal of oxygen at the metal surface through respiration by living cells within the biofilm. This protective effect has been shown for mild steel (Jayaraman et al. 1997a, b, c), copper, and aluminum (Jayaraman et al. 1999c). Jayaraman et al. (1999b) also used genetically engineered and non-engineered biofilms (Jayaraman et al. 1999a) to generate antimicrobials in situ to inhibit the growth of SRB and hence decrease the corrosion rate of metals. In this paper, we used biofilms to generate the antimicrobial gramicidin-S to protect mild steel from corrosive attack by both *D. orientis* and *L. discophora* SP-6. This is the first report of the inhibition of corrosion caused simultaneously by two different corrosion-causing bacteria.

Materials and methods

Bacterial strains, growth media, culture conditions, and chemicals

With the exception of *B. brevis* 18-3 (obtained from Prof. A.L. Demain, Massachusetts Institute of Technology) and *L. discophora* SP-6 ATCC 51168 (provided by Prof. Z. Lewandowski, Montana State University, Bozeman, Mont.), the bacteria were purchased from the American Type Culture Collection (Manassas, Va.). *B. brevis* 18-3, producing gramicidin-S (Azuma et al. 1992), and *Paenibacillus polymyxa* ATCC 10401 (previously *B. polymyxa* 10401) were grown from −80°C glycerol stocks in 250-ml shake-flasks with 25 ml LB medium (Maniatis et al. 1982) at 37°C and with shaking at 250 rpm (series 25 shaker; New Brunswick Scientific, Edison, N.J.).

D. orientis ATCC 23598 (Gram-positive SRB, formerly *Desulfovibrio orientis*) was cultivated as described by Jayaraman et al. (1999b). *L. discophora* SP-6 ATCC 51168 was inoculated from solid plates and grown in ATCC 1503 medium at 30°C with shaking at 250 rpm. Solid agar plates were used to determine the *L. discophora* cell number. Routine maintenance of this bacterium included streaking the cells on solid agar plates containing the same nutrients as the liquid medium, incubating at 30°C, and storing the Parafilm-wrapped plates at 4°C. Stock cultures were made by adding 15% glycerol to the liquid culture and storing at −80°C.

To facilitate the growth of both corrosion-causing bacteria (*D. orientis*, *L. discophora* SP-6) when they were inoculated in the same reactor, a special medium (DL medium) was formulated containing the required components for the growth of both bacteria. DL medium is a mixture of modified Baars' medium and ATCC 1503 medium and contains (per liter of deionized water): 0.5 g Bacto tryptone, 0.5 g yeast extract, 0.5 g glucose, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.07 g $CaCl_2 \cdot 2H_2O$, 0.017 g $MnSO_4 \cdot H_2O$, 3.57 g HEPES, 5 g sodium citrate, 1 g NH_4Cl, 1.26 g $CaSO_4 \cdot 2H_2O$, 0.5 g K_2HPO_4, and 5.84 ml of 60% sodium lactate syrup, pH 7.4. Gramicidin-S (96.5% purity) was purchased from Sigma Chemical Co., St Louis, Mo.

Metal coupon preparation for testing and post-test examination

Mild steel 1010 (UNS G10100) coupons of 10 cm^2 (1.2 mm thick) were cut from sheet stock (Yarde Metals, Bristol, Conn.) and degreased on one side with acetone. The degreased sides of the coupons were polished with 240 grit polishing paper (Buehler, Lake Bluff, Ill.) and rinsed with distilled water. After cleaning, the metal coupons were immediately wiped with a paper towel, then dried in an oven at 80°C for 10 min and cooled to measure mass loss (mg). At the end of the continuous experiments, the mass loss of each coupon was determined by cleaning the metal surface under a stream of tap water and scrubbed vigorously with a rubber stopper to remove corrosion products or biofilms. The mass loss of mild steel samples due to corrosion was measured using the chemical cleaning methods described in ASTM standard G1-90 (ASTM 2000).

Antimicrobial assay with pure gramicidin-S

The most probable number (MPN) method (Anonymous 1992) was used to determine the survival rate of *D. orientis*, as described by Jayaraman et al. (1999b) in the presence of various concentrations of gramicidin-S. A similar method was used to determine the viable percentage of *L. discophora* SP-6 with gramicidin-S.

After growing in liquid medium (ATCC 1503 medium) for about 6–7 days, 1 ml of *L. discophora* culture was centrifuged, washed with fresh ATCC 1503 medium, resuspended in fresh ATCC 1503 medium, and exposed to different concentrations of gramicidin-S solution. After 1 h of contact, the culture was diluted serially and spread on three solid agar plates for each dilution. The number of plates showing growth in each dilution set upon 3–4 days of incubation at 30°C was used as an index to obtain the MPN and determine the effect of gramicidin-S on the growth of *L. discophora* SP-6.

Scanning electron microscope and confocal microscopy

After the biofilm and corrosion products were removed by a rubber stopper, several metal coupons were examined (without destroying the metal surface) by scanning electron microscopy (SEM; model 2020; Philips Electronic Optics, Eindhoven, the Netherlands) to analyze the surface upon exposure to corrosion-causing bacteria. They were then chemically cleaned to determine the mass loss. Confocal microscopy (model TCS SP2; Leika, Heidelberg, Germany) was used to measure the biofilm thickness (Ren et al. 2002).

Continuous-culture corrosion experiments

Autoclavable continuous reactors consisting of a 6.2 cm diameter glass cylinder on top of the metal sample (sealed by an O-ring) and a Teflon top with fittings for electrodes and media flow was used as described by Jayaraman et al. (1997a). The working volume of the reactor was 150 ml, with an airflow rate of 200 ml/min (monitored with a FM1050 series flow meter; Matheson Gas Co., Cucamonga, Calif.). The growth temperature was maintained at 30°C by heating tape wrapped around the reactor. DL medium was continuously fed to the reactors at a flow rate of 12 ml/h using a Masterflex precision standard drive with a ten-turn potentiometer (Cole-Parmer, Niles, Ill.). Impedance data were recorded immediately after the continuous operation was started. Most of the continuous experiments were conducted at least in duplicate.

An overnight culture of protective, biofilm-forming *B. brevis* 18-3 or the biofilm-forming control *P. polymyxa* 10401 [optical density at 600 nm (OD_{600}) × volume (ml) = 10] was inoculated into continuous reactors in batch mode and each species was allowed to develop a biofilm on the metal surface for 2–3 days. *P. polymyxa* 10401 was used as a biofilm-forming control, since it did not inhibit the growth of *D. orientis* and the related corrosion on mild steel. Next, a 3% (v/v) *D. orientis* inoculum (culture age 3–4 days, OD_{600} = 0.13–0.16) either alone or with 20 ml of a 6–7-days *L. discophora* SP-6 culture was added to the continuous reactors just before continuous operation was initiated. *P. polymyxa* 10401 and *D. orientis* were also tested individually in separate sterile reactors to determine their effect on the corrosion of mild steel. To determine the baseline corrosion rate of mild steel in DL medium without a biofilm, mild steel was exposed to DL medium in continuous reactors with 50 μg/ml of kanamycin added to maintain sterility.

Electrochemical impedance spectroscopy

Biofilm development and corrosion rates during the continuous reactor experiments were monitored using electrochemical impedance spectroscopy (EIS), as described by Jayaraman et al. (1997a) and Örnek et al. (2002a). EIS is a non-destructive corrosion monitoring technique that can be used to study the corrosion behavior of the metal under investigation without disturbing the properties of the biofilms and metal surface (Mansfeld 1995; Jayaraman et al. 1999a). The EIS data were obtained at the corrosion potential (E_{corr}), using an IM6 impedance spectrometer (Bioanalytical Systems–Zahner, West Lafayette, Ind.) interfaced to a Pentium GP6 300 MHz computer (Gateway, North Sioux, S.D.) running THALES impedance measurement and equivalent circuit synthesis/simulation/fitting software. The experimental EIS spectra were analyzed to yield the polarization resistance (R_p), using an equivalent circuit (EC) proposed by Mansfeld et al. (1992) to describe the impedance behavior of materials undergoing uniform corrosion. This EC consists of R_p in parallel with the electrode capacitance C and in series with the solution resistance R_s. The corrosion rate can be determined by using the Stern–Geary equation: $I_{corr} = B/R_p$, where B is a system-dependent experimental parameter, and I_{corr} is the corrosion current density which can be converted to a corrosion rate using Faraday's law (Mansfeld 1976). Hence, the mild steel corrosion rate is inversely proportional to R_p. Since experimental values of B were not available, relative corrosion rates are used here, expressed as $1/(R_pA)$, where A is the area of the metal exposed to medium in the reactor. Integration of $1/(R_pA)$ versus time curves results in the parameter INT (units are seconds per ohm per square centimeter) which is proportional to the total mass loss of the test sample during the test period and which indicates the relative corrosion rate. Weight loss data (mg) were also measured in units of μm/year by dividing the experimental results by the electrode area (30.58 cm^2, the surface area of the tested metal exposed to the medium), the length of the experiments (year), and the density of mild steel (7.85 g/cm^3).

Results

Antimicrobial assay with pure gramicidin-S

Our previous results (Zuo et al. 2003a) showed that pure gramicidin-S was very effective at inhibiting the growth of both Gram-positive SRB (*D. orientis*) and Gram-negative SRB (*D. vulgaris*), with 95.4% of *D. orientis* killed at

1 μg/ml gramicidin-S and 93.5% of *D. vulgaris* killed at 100 μg/ml gramicidin-S, which led us to test the ability of using this antimicrobial to inhibit another corrosion-causing bacterium, *L. discophora* SP-6. Our results indicate gramicidin-S is effective in inhibiting *L. discophora* SP-6 at a concentration of 20 μg/ml (97% of cells killed).

Baseline corrosion rate of mild steel in DL medium and the effect of the control biofilm on the corrosive bacterium *D. orientis*

Our previous study investigated the effect of modified Baars' medium and TMI water on the corrosion rate of mild steel in the presence of a Gram-positive SRB (*D. orientis*; Zuo et al. 2003a). In the present study, a new medium (DL medium) was formulated for the growth of both SRB (*D. orientis*) and the iron-oxidizing bacterium, *L. discophora* SP-6. To determine the effect of this new medium on the corrosion of mild steel in the presence of either *D. orientis* alone or both corrosion-causing bacteria together, it was necessary to measure the baseline corrosion rate of mild steel in this medium. When no bacterium was present in DL medium (sterile control), the corrosion rate was higher than the corrosion rate when the non-gramicidin-producing *P. polymyxa* ATCC 10401 biofilm control was present on the metal surface, as shown by the impedance data (1.7-fold higher corrosion rate represented by INT parameter values) and mass loss data (2.6-fold higher; Table 1). The protection afforded by the *P. polymyxa* ATCC 10401 biofilm on mild steel relative to the sterile control is expected, since biofilms on metal surfaces can exclude oxygen at the metal surface and thereby reduce corrosion (Jayaraman et al. 1997a). During the experiments with *D. orientis* alone, H_2S was generated after 1 day of continuous operation, indicating the robust growth of this strain (Jayaraman et al. 1999a), and after the experiments were finished, a thin black film could be seen on the metal surface, which was removed by a rubber stopper, leaving a gray surface after washing. When only the *P. polymyxa* ATCC 10401 biofilm was present, the metal surface remained clean during the 13-day test. Compared with the *P. polymyxa* ATCC 10401 control biofilm, *D. orientis* caused more corrosion in terms of the corrosion rate (1.2-fold difference, as shown by INT values) and mass loss (1.9-fold difference; Table 1).

Inhibition of both corrosion-causing bacteria, *D. orientis* and *L. discophora* SP-6

In these two sets of continuous-culture corrosion experiments, *P. polymyxa* ATCC 10401 was introduced into the reactors first, to establish a biofilm before the corrosion-causing bacteria were inoculated. The presence of the *P. polymyxa* ATCC 10401 biofilm (the biofilm control) on the metal surface promoted the growth of *D. orientis*, as indicated by the medium turning black on the same day when *D. orientis* was inoculated, 1 day earlier than when no biofilm of *P. polymyxa* ATCC 10401 was present; and a thick black film was observed on the metal surface at the end of the experiment. Accordingly, the corrosion caused by *D. orientis* increased when the *P. polymyxa* 10401 biofilm was present, with the EIS-based INT values showing a 2.2-fold difference in the corrosion rate but not much difference for the mass loss data (Table 1). Furthermore, contrary to the corrosion rate of mild steel, which decreased with time when only *D. orientis* was present, the corrosion rate of mild steel increased for the first 7 days and remained high when both *P. polymyxa* 10401 and *D. orientis* were present, since the oxygen consumed by the *P. polymyxa* 10401 biofilm helped to create an anaerobic environment favoring the growth of the anaerobic *D. orientis* and subsequent anaerobic corrosion.

The iron-oxidizing bacterium *L. discophora* SP-6 itself did not cause significant corrosion on mild steel in ATCC 1503 medium when present by itself or combined with the biofilm control *P. polymyxa* 10401, as indicated by the comparatively high impedance data and low mass loss data (data not shown). But when *L. discophora* SP-6 was combined with another corrosion-causing bacterium, *D. orientis* in the presence of *P. polymyxa* 10401, the corrosion of mild steel increased significantly compared with that when only *D. orientis* was included in the

Table 1 Corrosion of mild steel 1010 in continuous experiments in the presence of corrosion-causing bacteria (*D. orientis*, *L. discophora* SP-6). *P. polymyxa* 10401 is a biofilm-forming, non-antibiotic-producing negative control. The corrosion rate is obtained by dividing the experimental results of mass loss by the electrode area (30.58 cm^2, the surface area of the tested metal exposed to the medium), the length of the experiments (year), and the density of mild steel (7.85 g/cm^3). Mass loss is shown after 12–13 days. The INT parameter is the integral of $1/(R_pA)$ over time and is proportional to the amount of corrosion. The corrosion potential, E_{corr}, is the average value over 12–13 days. Data are from at least two replicated experiments

Protective biofilm	Corrosion bacteria	Corrosion rate (μm/year)	Mass loss (mg)	EIS-based INT parameter	E_{corr} (mV vs Ag/AgCl)
B. brevis 18-3	*D. orientis* and *L. discophora* SP-6	25±10	22±8	20±2	−400±30
P. polymyxa 10401		36±4	29±4	66±7	−390±70
P. polymyxa 10401	*D. orientis*	60±10	48±8	173±54	−440±30
No protective biofilm	*D. orientis*	71±3	56±3	77±11	−450±40
Sterile		93±10	74±8	110±46	−830±70
P. polymyxa 10401	*D. orientis* and *L. discophora* SP-6	111±11	87±9	404±137	−460±40

reactors with *P. polymyxa* 10401, as evidenced by a 2.3-fold higher corrosion rate shown by the EIS-based INT values and a 1.8-fold higher mass loss (Table 1). This increase in corrosion rate was also accompanied by the enhanced growth of *D. orientis*, as evidenced by the very strong odor of H_2S, the black medium in the reactors, and a very thick and sticky black film formed on the mild steel surface when the experiments were stopped.

Since pure gramicidin-S inhibited both *D. orientis* and *L. discophora*, a *B. brevis* 18-3 biofilm was used to produce gramicidin-S from within the biofilm to inhibit both corrosion-causing bacteria simultaneously. During the continuous-culture corrosion experiments with the *B. brevis* 18-3 biofilm, no H_2S odor was detected and the medium and the metal surface did not turn black (qualitatively indicating no growth of SRB). Based on the EIS-based INT values, the protective biofilm decreased the corrosion rate about 20-fold compared with the *P. polymyxa* 10401 biofilm control and the mass loss for the protective biofilm was also significantly lower than that for the biofilm control, with about a 4-fold difference (Table 1). This protection achieved by the *B. brevis* 18-3 biofilm was not due to the removal of oxygen (which also occurred with *P. polymyxa* 10401 and the two corrosion-causing bacteria in its biofilm), but due to the gramicidin-S produced by the *B. brevis* 18-3 biofilm and its inhibition of both *D. orientis* and *L. discophora* SP-6.

The relative average mass loss for all the continuous-culture corrosion experiments is summarized in Table 1. It is clear that the mild steel sample exposed to both deleterious bacteria in the presence of the biofilm control suffered the most severe corrosion damage, while the gramicidin-S-producing *B. brevis* 18-3 biofilm offered significant protection against this attack.

The corrosion potential for mild steel exposed to the sterile DL medium decreased by 200 mV with time. In addition, the E_{corr} in sterile medium was much more negative than that for mild steel exposed to the different biofilms. Ennoblement due to the presence of biofilms was observed, with an increase of 200–400 mV (Table 1). This ennoblement in the presence of biofilms has been reported for aluminum and brass (Örnek et al. 2001, 2002b, c), which showed an increased E_{corr} in the range of 100–400 mV in the presence of different biofilms and in different media. Based on these observations, Mansfeld et al. (2002) suggested that ennoblement is common for protective biofilms that might produce inhibitive species, which are responsible for the corrosion reduction. Our hypothesis is that the negatively charged bacterial walls and extracellular polymeric substances in the biofilm might be responsible for the ennoblement by repelling anions in the solution.

SEM metal surface analysis

The protection of mild steel afforded by the gramicidin-S-producing *B. brevis* 18-3 biofilm against corrosion caused by the simultaneous addition of *D. orientis* and *L. discophora* SP-6 was corroborated by SEM. Figure 1 shows the surface appearance of polished mild steel at a magnification of 1,000× after 13 days of continuous-culture corrosion experiments. Figure 1a shows that the surface of the sample protected by the *B. brevis* 18-3 biofilm, when exposed to *D. orientis* and *L. discophora* SP-6, still contains the polishing grooves. However, these grooves are not visible on the surface of the metal in the presence of biofilm control *P. polymyxa* 10401 when exposed to *D. orientis* and *L. discophora* SP-6 (Fig. 1b). Hence, the gramicidin-S-producing *B. brevis* 18-3 biofilm thwarted the simultaneous attack by *D. orientis* and *L. discophora* SP-6. Note that confocal microscopy showed that both *B. brevis* and *P. polymyxa* formed comparable biofilms (thicknesses of 17±3 μm for *B. brevis*, 13±3 μm for *P. polymyxa*).

Discussion

In natural ecosystems, the activity of SRB is not the only reason for microbiologically induced corrosion (Licina 1988; Hamilton 1990). As an obligate anaerobic organism, SRB growth is affected by the activities of other organisms in the same niche (Geesey 1990; Hamilton 1990). The

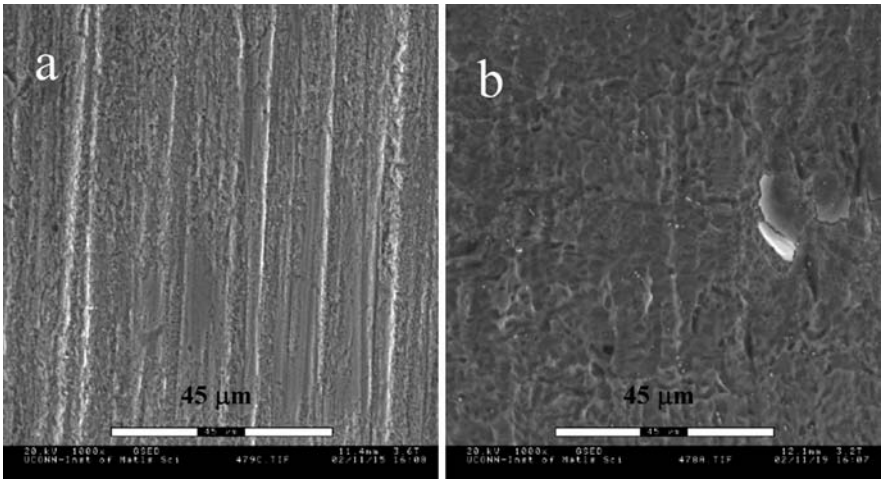

Fig. 1 **a,b** SEM images (1,000×) of mild steel 1010 exposed to both *D. orientis* and *L. discophora* SP-6 after 12–13 days in DL medium in continuous experiments. **a** Mild steel 1010 coupon exposed to both *D. orientis* and *L. discophora* SP-6 was protected by a *B. brevis* 18-3 biofilm. **b** Mild steel 1010 coupon exposed to *D. orientis* and *L. discophora* SP-6 was not protected by *P. polymyxa* 10401 (biofilm control). *Bar* 45 μm

aerobes consume oxygen by respiration, creating a local anaerobic environment in the biofilm, as required by the SRB. In addition, some fermentative facultative anaerobes provide organic electron donors for SRB to obtain energy (Geesey 1990). Thus, it is not unexpected that the coexistence of two different biofilm-forming microorganisms, like aerobic *P. polymyxa* 10401 and anaerobic SRB, accelerates the corrosion of the metal beneath them, resulting in higher corrosion rates than when only *D. orientis* is present (2.2-fold higher, as shown by INT data in Table 1), since the *P. polymyxa* 10401 biofilm creates an anaerobic environment, stimulating the growth of *D. orientis* and leading to a more severe attack by *D. orientis*.

It is unlikely that, in a natural environment, metal surfaces are colonized by only one corrosion-causing bacterium (Licina 1988; Rao et al. 2000). Instead, different species of microorganisms grow in close proximity, forming heterogeneous biofilms (Geesey 1990). The sheath-forming *Leptothrix* genus is one of the most common iron-oxidizing bacteria (Rao et al. 2000), causing the tuberculation of the pipelines in cooling water systems (a process which makes a localized tubercle containing deposits of corrosion products, cells, and metabolites on the metal surface; Licina 1988). This genus might cause severe safety problems, especially in nuclear power plants, since this organism forms tubercles which blocks the pipes and significantly decreases the flow rate of cooling water supplying key equipment, resulting in possible failure of temperature control and the possibility of subsequent explosions (Rao et al. 2000).

Rao et al. (2000) described the prevalence of iron-oxidizing bacteria and SRB in the cooling circuit of a nuclear test reactor and how they were involved in the corrosion of carbon steel in this cooling system. Their observations revealed that the corrosion rate of carbon steel reached a maximum value when the maximum population of the iron-oxidizing bacteria appeared on carbon steel coupons.

Heterogeneous distribution of microorganisms on metal surfaces can cause the formation of differential aeration cells (Geesey 1990) which are due to the difference in oxygen concentration between the area covered by oxygen-respiring bacteria (or oxygen-impervious tubercles) and the area exposed to the bulk liquid phase. In a differential aeration cell system, the metal surface beneath the biofilms and mineral precipitates has a low redox potential and acts as the anode, resulting in dissolution of the metal (Geesey 1990). In our experiments, the addition of *L. discophora* SP-6 increased the corrosion rate of mild steel in the presence of SRB (*D. orientis*), and one possible explanation is that the precipitated iron-storing sheath of the iron-oxidizing bacterium *L. discophora* forms an oxygen-impervious membrane (Rao et al. 2000), creating an anaerobic environment which promotes the growth of SRB (*D. orientis*). Meanwhile, as the sheaths grow thicker, the difference in potential between the metal surface beneath and the outside of the tubercle increases, making the metal surface beneath the sheaths an anode relative to that exposed to the aerated bulk liquid phase (Hamilton 1990), and both of these phenomena accelerate the corrosion rate of the metal (Rao et al. 2000). Thus, our experimental results clearly show that the existence of multiple corrosion-causing bacteria is synergistic and contributes to the corrosion of the attached metals being much more severe than when only one single corrosion-causing bacterium is present. However, when both corrosion-causing bacteria are killed by gramicidin-S produced in situ by the *B. brevis* 18-3 biofilm, the oxygen-impervious membrane and anode sheath are not formed, so corrosion is not accelerated and a dramatic reduction in corrosion is seen, as compared with the *P. polymyxa* 10401 biofilm control, which is unable to prevent the growth of *D. orientis* and *L. discophora*. The current system therefore provides an alternative for controlling microbiologically influenced corrosion that may be applicable to industrial systems.

Acknowledgements We thank Prof. F. Mansfeld and Mr. Chao-Hung Hsu of the University of Southern California for modeling some of the EIS data. This project is supported by the Electric Power Research Institute (contract EP-P5388-C2665).

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