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ORIGINAL PAPER

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Inhibiting mild steel corrosion from sulfate-reducing bacteria using antimicrobial-producing biofilms in Three-Mile-Island process water

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Abstract Biofilms were used to produce gramicidin S (a cyclic decapeptide) to inhibit corrosion-causing, sulfate-reducing bacteria (SRB). In laboratory studies these biofilms protected mild steel 1010 continuously from corrosion in the aggressive, cooling service water of the AmerGen Three-Mile-Island (TMI) nuclear plant, which was augmented with reference SRB. The growth of both reference SRB (Gram-positive *Desulfosporosinus orientis* and Gram-negative *Desulfovibrio vulgaris*) was shown to be inhibited by supernatants of the gramicidin-S-producing bacteria as well as by purified gramicidin S. Electrochemical impedance spectroscopy and mass loss measurements showed that the protective biofilms decreased the corrosion rate of mild steel by 2- to 10-fold when challenged with the natural SRB of the TMI process water supplemented with *D. orientis* or *D. vulgaris*. The relative corrosion inhibition efficiency was 50–90% in continuous reactors, compared to a biofilm control which did not produce the antimicrobial gramicidin S. Scanning electron microscope and reactor images also revealed that SRB attack was thwarted by protective biofilms that secrete gramicidin S. A consortium of beneficial bacteria (GGPST consortium, producing gramicidin S and other antimicrobials) also protected the mild steel.

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Introduction

Sulfate-reducing bacteria (SRB), such as *Desulfovibrio* species and *Desulfomaculum* species, cause corrosion of cast iron, carbon and low alloy steels, stainless steels, high nickel alloys and copper alloys (Licina 1988). U.S. losses as a result of SRB corrosion damage are estimated to be $4–6 billion/year (Beloglazov et al. 1991). Microbiologically influenced corrosion (MIC) mostly occurs under stagnant conditions or in operations with low or intermittent flow of river or sea water (Licina 1988) and is problematic for the nuclear power plant, paper-making, and oil industries (Miller 1981; Licina 1988). MIC can cause considerable damage to cooling water systems (Borenstein 1994), sewage treatment facilities (Odom 1990), underground pipes (Miller 1981), and ships at low tide (Miller 1981). Black FeS films on stainless steel or mild steel are generally indicative of SRB attack, and when they are lifted, pits are revealed (Miller 1981).

Biocide treatments are widely used to decrease biofouling and MIC in steel pipes (Franklin et al. 1991) and in closed systems (Fontana 1986). Cathodic protection is also used successfully to prevent MIC when used with coatings (Fontana 1986). However, the use of biocides and cathodic protection techniques are very expensive for industry (Pankhurst 1968; Cord-Ruwisch et al. 1987). Biocides can also cause environmental pollution (Cord-Ruwisch et al. 1987), and if they are used at concentrations in excess of a few ppm, they can be corrosive to metals (Franklin et al. 1991). MIC occurs under adherent biofilms (Costerton et al.1987). Biocides are not as effective against sessile organisms within biofilms as against a planktonic population (Hamilton 1985).

Some biofilms have been shown to be beneficial for reducing corrosion damage (Potekhina et al. 1999; Little and Ray 2002), and new strategies have been developed to combat the colonization of SRB from within the biofilm itself. Our laboratory (Jayaraman et al. 1999a) has identified novel biofilms that inhibit the development of SRB on metal surfaces, such as mild steel and stainless steel (only *Desulfovibrio vulgaris* was tested). We also genetically engineered an aerobic, biofilm-forming *Bacillus* bacterium to produce antimicrobials, such as indolicidin and bactenecin, which was effective in preventing the SRB *D. vulgaris* from colonizing the metal surfaces (Jayaraman et al. 1999b).

Since a recent review suggested that little work has been performed using protective biofilms in service water [most experiments have been performed in laboratory medium (Little and Ray 2002)], the present study was conducted and is the first report evaluating the impact of antimicrobial-producing biofilms against SRB in industrial service water (and the first report of the inhibition of the SRB *Desulfosporosinus orientis* by an antimicrobial-producing biofilm). MIC has been identified at the AmerGen Three-Mile-Island (TMI) nuclear plant, and visual examination of damaged pipes in the plant has shown thick, black slimy deposits or films on the surface of pipes exposed to the TMI process water. Leaks due to pitting are a common problem in the region, and the organisms identified in this region are sulfate-reducing and sulfur- and iron-oxidizing bacteria. In this study, antimicrobial-producing bacteria were used to form a protective biofilm on mild steel, and it was found that these protective biofilms prevented the growth of the SRB present in the TMI process water as well as of the SRB that were added (*D. vulgaris* and *D. orientis*). These biofilms secreted the antimicrobials gramicidin, gatavalin, polymyxin, subtilin, and tyrocidine in continuous reactors.

Materials and methods

Bacterial strains, plasmids, growth media, and culture conditions

With the exceptions of *Bacillus brevis* Nagano and *Bacillus brevis* 18–3 (obtained from Prof. A. L. Demain, Massachusetts Institute of Technology), and *Bacillus subtilis* WB600 (obtained from Dr. Sui-Lam Wong of the University of Calgary), the bacteria were purchased from the American Type Culture Collection (ATCC, Manassas, Va.).

B. subtilis WB600 (Wu et al. 1991) is a protease-deficient strain and was used as a biofilm control for *D. vulgaris* since it produces a biofilm but does not produce antimicrobials against this Gram-negative SRB. However, *B. subtilis* WB600 inhibited the growth of *D. orientis*, therefore *Paenibacillus polymyxa* 10401 was used as a biofilm control for the experiments with this Gram-positive SRB since it did not inhibit the growth of *D. orientis* in batch experiments. The following strains were used in batch supernatant experiments: *B. brevis* Nagano [producing gramicidin S (Azuma et al. 1992)], *B. brevis* 18–3 [producing gramicidin S (Azuma et al. 1992)], *B. parabrevis* ATCC 10068 (previously *B. brevis* 10068, producing gramicidin and tyrocidine), *Aneurinibacillus migulanus* ATCC 9999 (previously *Bacillus migulanus* 9999, producing gramicidin S), *P. polymyxa* ATCC 10401 (previously *Bacillus polymyxa* 10401, producing polymyxin) and *B. subtilis* ATCC 6633 (producing subtilin). The antimicrobial GGPST consortium (gramicidin-gatavalin-polymyxin-subtilin-tyrocidine) consists of *B. brevis 18–3*, *B. brevis* Nagano, *B. subtilis* ATCC 6633, *P. polymyxa* ATCC 21830 (previously *Bacillus polymyxa* 21830, producing gatavalin*), B. parabrevis* 10068, *Bacillus circulans* ATCC 31228 (producing polymyxin F), and *A. migulanus* ATCC 9999.

A biofilm of *P. polymyxa* 10401 with either plasmid pBE92-Bac or plasmid pBE92-Probac was introduced to inhibit corrosion-causing SRB by producing and secreting the additional antimicrobial polypeptides bactenecin or probactenecin (Jayaraman et al. 1999b). The pBE92 plasmid makes the biofilm resist 50–100 μg/ml kanamycin. The amino acid sequence for bactenecin is NH_2-Arg-Leu-Cys-Arg-Ile-Val-Val-Ile-Arg-Val-Cys-Arg-OH (Romeo et al. 1988). Plasmid pBE92-Bac (Jayaraman et al. 1999b), a derivative of pBE60 (Nagarajan et al. 1992), was designed to express bactenecin as a 13 amino acid peptide fused to the *apr* signal sequence (Chen and Nagarajan 1993), and pBE92-ProBac was designed to express bactenecin fused to the pro-region of the extracellular RNase barnase from *Bacillus amyloliquefaciens* (Paddon et al. 1989) and the *apr* signal sequence.

D. orientis ATCC 23598 (Gram-positive SRB, formerly *Desulfovibrio orientis*) and *D. vulgaris* ATCC 29579 (Gram-negative SRB) were cultivated as described previously (Jayaraman et al. 1999b). Into sterile, long cylindrical tubes with black screw caps (15 ml), 13 ml of modified Baars' medium (ATCC medium 1249), 260 μl sodium sulfite, and 260 μl oxyrase (Oxyrase Inc., Mansfeld, Ohio) were added. After 30 min, 1 ml of a thawed −80°C glycerol stock of the SRB (e.g., *D. orientis*) culture was added, the tubes closed, and incubated at 30°C without shaking for 3–5 days. For the antimicrobial-producing bacteria, overnight cultures 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.).

TMI process water was received from the AmerGen Three-Mile-Island Nuclear Generation Station, Middletown, Pa. It is used in the circulation water system to condense the spent (low energy) steam from the main turbines. The source of the process water is the Susquehanna River (16–32°C in winter, 32–49°C in summer). It is characterized as aggressive in terms of causing mild steel corrosion of 1.65 mm per year (1.65 mm of wall loss per year, pitting rate measured at TMI in the circulating water system, and this value varies from system to system, unpublished). There are about 50,000 m^3 (13 million gallons) in the total system with 38–45 m^3/min (10,000–12,000 gal/min) make-up from the river. The make-up volume is the direct result of losses in the cooling towers (moisture carryover and some evaporation) and results in a significant concentration effect; hence, there is a high concentration of Mg^{2+} and Ca^{2+} along with other minerals in the TMI service water (2,000–4,000 ppm). There is a small blowdown flow back to the river to aid in lowering the mineral concentration.

Metal coupon preparation for testing and post-test examination

Mild steel 1010 coupons (UNS G10100, 1.2 mm thick, 2.5×2.5 cm or 10×10 cm) 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. At the end of the experiments, the metal surfaces were cleaned under a stream of tap water and scrubbed vigorously with a rubber stopper to remove corrosion products or biofilms. After cleaning, the metal plates were immediately wiped with a paper towel, then dried in an oven at 80°C for 10 min and cooled to measure the mass loss (mg).

Scanning electron microscope

After the mass-loss measurements were completed, the metal plates were cut into 2.5-cm squares, dehydrated with ethanol, and dried at 80°C for 15 min. The samples were coated with an Au-Pd alloy and

examined with scanning electron microscopy (SEM 2020, Philips Electronic Optics, Eindhoven, Netherlands).

Batch experiments with supernatants of antimicrobial-producing bacteria

Supernatants (25 ml) of overnight antimicrobial-producing bacteria cultures were filter-sterilized and mixed with an equal volume of modified Baars' medium in a 250 ml flask containing a 2.5×2.5 cm mild steel coupon. The resulting solution was inoculated with a 3-day old *D. orientis* culture (OD_{600}=0.16, 3% v/v). After 21 days of static anaerobic incubation at 30°C, the mass loss of the mild steel coupon was measured. As a control, 25 ml of sterile LB or sterile modified Baars' medium, instead of supernatant from the antimicrobial-producing bacteria cultures, was mixed with an equal volume of Baars' medium. All mass-loss data were an average of three samples.

Antimicrobial assay with pure gramicidin S

A 3–5-day old culture of SRB (*D. orientis* or *D. vulgaris*, OD_{600} = 0.2–0.3) was taken and 1.5 ml centrifuged. The precipitated cells were washed with fresh modified Baars' medium and resuspended in 1.35 ml of fresh modified Baars' medium supplemented with 4% Na_2SO_3 and oxyrase. The suspended cells were then incubated with 0–120 μg/ml gramicidin S for 1 h. To check the survival rate of the SRB, the samples were serially diluted by adding 1.5 ml of the culture into 13.5 ml of fresh modified Baars' medium (supplemented with 4% Na_2SO_3 and oxyrase) and incubated anaerobically at 30°C. After 3–5 days, 1 ml of 5% $Fe(NH_4)_2(SO_4)_2$ was added to the tubes to see if a black precipitate appeared, indicating growth of SRB (Jayaraman et al. 1999a). The number of tubes showing growth in each dilution set was used as an index to obtain the most probable number (MPN) (Anonymous 1992) of SRB cells at this dilution rate and to determine how efficiently gramicidin S inhibited the growth of SRB. Gramicidin S (96.5% purity) was purchased from Sigma, St Louis, Mo., USA.

Continuous culture corrosion experiments

Autoclavable continuous reactors consisting of a 6.2 cm diameter glass cylinder on top of the metal sample were used as described previously (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 flowmeter, Matheson Gas Company, Cucamonga, Calif.). The growth temperature was maintained at 30°C by heating tape wrapped around the reactor. The TMI water used in each experiment was fed at a nutrient flowrate of 12 ml/h using a Masterflex precision standard drive with a 10-turn potentiometer (Cole-Parmer, Niles, Ill.); hence, the dilution rate was 0.08/h. Most of the continuous experiments were conducted at least in duplicate (see figure captions).

For continuous experiments with TMI water (no nutrients), LB medium was added to the reactors initially to establish the protective biofilms (one time addition of LB) while TMI water was in the feed bottles; for continuous experiments with modified Baars' medium, Baars' medium was added into both the reactors and the feed bottles. Except where indicated, for the continuous culture corrosion experiments with SRB, an equal volume of inoculum was used for each reactor for both the SRB culture (usually 13–15 ml of a 3-day old SRB culture suspension, OD_{600}=0.12–0.3, to 3 l of TMI water in the feed bottle or 5 ml of SRB culture to 150 ml modified Baars' medium in the reactor) and the antimicrobial-producing bacteria (10 ml of overnight bacteria culture to 150 ml LB in the reactor). The TMI water containing the reference SRB or the modified Baars' medium in the feed bottles was either fed immediately to the reactors or after 1–2 days of batch operation in which the antimicrobial-producing bacteria were allowed to produce a protective biofilm.

For the TMI water control experiment, TMI process water containing SRB was added to the reactor after the sterile reactor containing LB medium had been operating for 2 days in batch mode. Since the TMI process water was not sterile, biofilms developed in the control experiment after the sterile batch period.

Electrochemical impedance spectroscopy

Biofilm development and corrosion rates were monitored during the continuous reactor experiments using electrochemical impedance spectroscopy (EIS) as described previously (Jayaraman et al. 1997a). EIS is a non-destructive corrosion monitoring technique that can be used to study 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 with a Gateway Pentium GP6 300 MHz computer (North Sioux, S.D.) running THALES Impedance Measurement and Equivalent Circuit Synthesis/Simulation/Fitting software. The experimental impedance spectra were analyzed to yield the polarization resistance (R_p) using an equivalent circuit (EC) that has been proposed by Mansfeld and co-workers to describe the impedance behavior of materials undergoing uniform corrosion (Mansfeld et al. 1992). This EC consists of the polarization resistance 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 $1/(R_p \cdot A)$, where A is the area of the metal exposed to medium in the reactor. The relative corrosion inhibition efficiency (E_{rel}) is defined as $E_{rel} = 100(1 - R_{p,con}/R_{p,bio})$, where $R_{p,con}$ and $R_{p,bio}$ refer to the polarization resistance (R_p) values determined in control experiments and in media containing protective bacteria, respectively. Integration of the $1/(R_p \cdot A)$ – time curves results in the parameter INT with the units of $s/(ohm \cdot cm^2)$, which is proportional to the total mass loss of the test sample during the test period. The relative corrosion inhibition efficiency can also be determined using INT data. Weight-loss data (mg) are also represented using the unit of μm/year by dividing the experimental results with the electrode area (30.58 cm^2, surface area of the tested metal exposed to the medium), the time of the experiments (year), and the density of mild steel (7.85 g/cm^3).

Results

Gramicidin S inhibits SRB

Supernatants from batch cultures producing gramicidin S were used to show that the peptide inhibits SRB. In the negative control experiments, the mild steel 1010 surface in flasks containing supernatants of *P. polymyxa* 10401, LB medium, or only Baars' medium turned black after 3 days, indicating robust growth of *D. orientis* (in these flasks the whole medium turned black in 4–5 days). The coupons became covered with thick dark corrosion products that were difficult to remove with a rubber stopper. In contrast, in flasks containing supernatants of the gramicidin-S-producing bacteria *B. brevis* 18–3, *B. brevis* Nagano, *B. parabrevis* 10068, *A. migulanus* 9999, or *B. subtilis* 6633, the metal coupons remained clean and

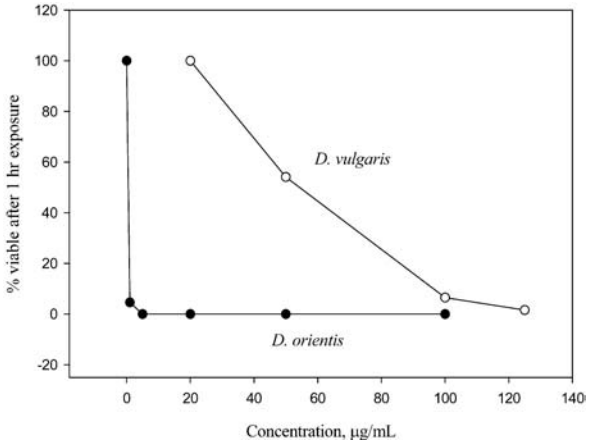
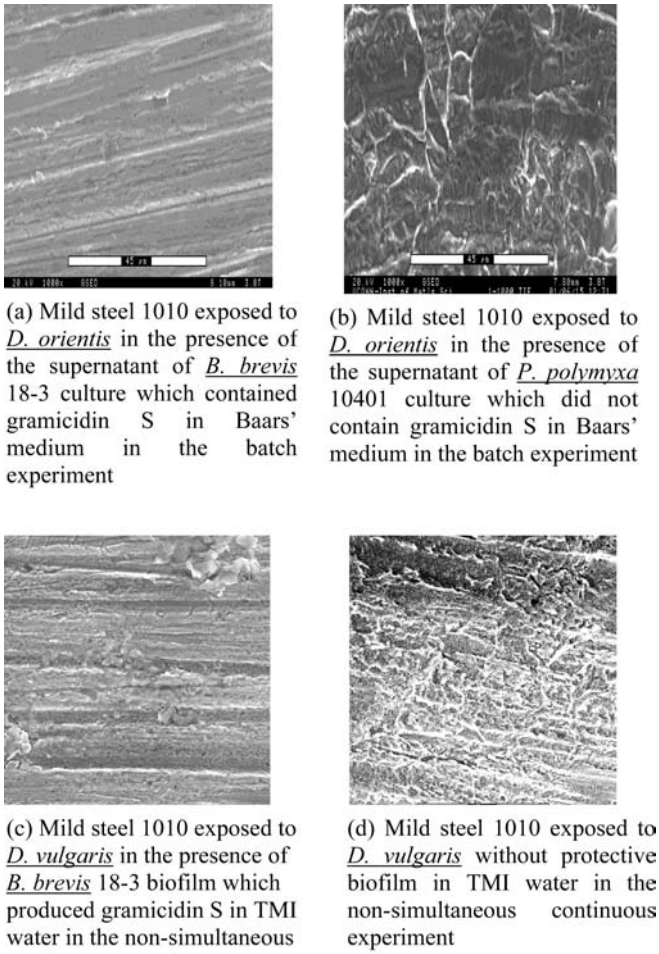


Fig. 1 Viability curves of *D. orientis* (*solid symbol*) and *D. vulgaris* (*open symbol*) upon exposure to gramicidin S for 1 h at 30°C. Cell numbers were determined by the most probable number method

shiny after washing under tap water, indicating inhibition of *D. orientis* growth. The mass loss data [0.33 (0.6) mg or 0.5 (0.9) μm/year for *B. brevis* 18–3, *B. brevis* Nagano and *B. parabrevis* 10068; 0.7 (0.6) mg or 1.1 (0.9) μm/year for *A. migulanus* 9999] showed that the supernatants of gramicidin-producing bacteria decreased the corrosion rates of mild steel by *D. orientis* about 19- to 40-fold compared to sterile LB medium [mass-loss data are 13 (2) mg or 21 (3) μm/year], 17- to 34-fold compared to sterile Baars' medium [mass-loss data are 11 (1) mg or 18 (2) μm/year], and 16- to 32-fold compared to the supernatant of *P. polymyxa* 10401 [mass-loss data are 11 (3.5) mg or 17 (6) μm/year]. *P. polymyxa* 10401 was then utilized as a biofilm control in later experiments since it was not effective against *D. orientis*.

In order to show that it was gramicidin S in the supernatants that was responsible for the inhibition of the SRB, an antimicrobial assay with pure gramicidin S was conducted. Both Gram-positive SRB (*D. orientis*) and Gram-negative SRB (*D. vulgaris*) were inhibited significantly, with 95.4% of *D. orientis* killed at 1 μg/ml and 93.5% of *D. vulgaris* killed at 100 μg/ml (Fig. 1).

The protection of mild steel afforded by the protective gramicidin-S-containing supernatants was corroborated by scanning electron microscopy (SEM). Fig. 2 (a and b) shows the surface appearance of polished mild steel with magnification 1000x after 21 days exposure to the above supernatants. Figure 2a shows that the surface of the sample exposed to *D. orientis* and the supernatant of *B. brevis* 18–3 for 21 days still contains the polishing grooves. However, these grooves are not visible on the surface of the metal exposed to *D. orientis* and the supernatant of *P. polymyxa* 10401, the biofilm control which does not produce gramicidin S (Fig. 2b). Hence, the extent of corrosion is much greater when the gramicidin-S-containing supernatant is not present.



(a) Mild steel 1010 exposed to *D. orientis* in the presence of the supernatant of *B. brevis* 18-3 culture which contained gramicidin S in Baars' medium in the batch experiment

(b) Mild steel 1010 exposed to *D. orientis* in the presence of the supernatant of *P. polymyxa* 10401 culture which did not contain gramicidin S in Baars' medium in the batch experiment

(c) Mild steel 1010 exposed to *D. vulgaris* in the presence of *B. brevis* 18-3 biofilm which produced gramicidin S in TMI water in the non-simultaneous continuous experiment

(d) Mild steel 1010 exposed to *D. vulgaris* without protective biofilm in TMI water in the non-simultaneous continuous experiment

Fig. 2 SEM images (1,000 ×) of mild steel 1010 exposed to *D. orientis* (**a, b**) in the presence of antimicrobial-containing supernatant in Baars' medium for 21 days, and to *D. vulgaris* (**c, d**) with or without a protective biofilm in TMI water in the continuous reactor experiment for 10 days. *Scale bar* is 45 μm in length

Continuous reactor inhibition of *D. orientis* in Baars' medium

Based on the success of inhibiting SRB with gramicidin S in batch experiments, a *B. brevis* 18–3 biofilm was used to protect mild steel against corrosion-causing *D. orientis* in Baars' medium in a continuous reactor with *P. polymyxa* 10401 as a biofilm control. Baars' medium is a good medium for culturing SRB (Jayaraman et al. 1999b). The protective biofilm was established on the metal surface prior to the addition of the SRB. Note that it was necessary to add SRB to augment the natural SRB in the TMI process water to get consistently active SRB growth.

In the presence of the non-protective *P. polymyxa* 10401 biofilm, the metal surface turned black in 1 day and smelled of H_2S after 3 days of continuous operation, indicating strong growth of *D. orientis*. However, in the presence of the protective *B. brevis* 18–3 biofilm, the metal surface did not turn black and there was no

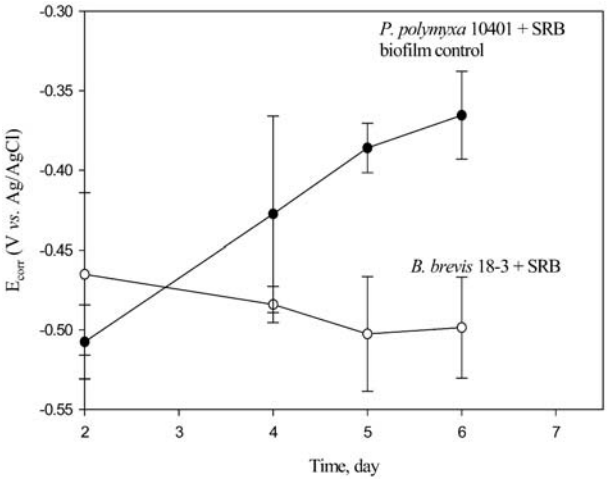


Fig. 3 Corrosion potential (E_{corr}) of mild steel 1010 in the presence of SRB (*D. orientis*) and *B. brevis* 18–3 (protective) or *P. polymyxa* 10401(control) biofilms in continuous reactors containing Baars' medium. *Solid symbols* represent *P. polymyxa* 10401 + SRB (duplicate), *open symbols* represent protective biofilm of *B. brevis* 18–3 + SRB (duplicate). *Error bars* shown for the standard deviations

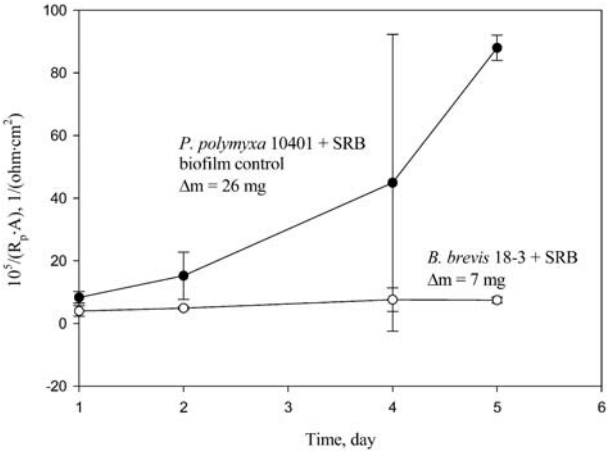


Fig. 4 Relative corrosion rates [$1/(R_p \cdot A)$] for mild steel 1010 in the presence of SRB (*D. orientis*) and *B. brevis* 18–3 (protective) or *P. polymyxa* 10401 (control) biofilms in continuous reactors containing TMI water. Symbols and error bars as defined in Fig. 3. Mass loss shown after 5 days.

evidence of H_2S generation during the 6 days of continuous operation. Although the impedance spectra could not be modeled because of the scatter of data in the low frequency region, the impedance spectra indicate that the protective biofilm of *B. brevis* 18–3 significantly decreased the corrosion rate of mild steel compared to the biofilm control (*P. polymyxa* 10401), which did not afford protection against *D. orientis* in Baars' medium (data not shown). The mass-loss data support the qualitative impedance results and demonstrate that inhibition of the SRB results in decreased corrosion rates. The protective biofilm *B. brevis* 18–3 reduced the corrosion rate 2.6-fold by decreasing the mass loss from 36 (4) μm/year [Δm=14 (1.4) mg] for the biofilm control to 14 (2) μm/year [Δm=5.5 (0.7) mg]. E_{corr} for the biofilm control was about 130 mV more positive than that for the protective biofilm at the end of the experiments (Fig. 3). This suggests that the oxygen concentration at the mild steel surface was lessened in the presence of the protective biofilm, resulting in a decrease of the rate of the cathodic reaction and therefore also of the corrosion rate.

Continuous reactor inhibition of *D. orientis* in TMI process water

The purpose of these experiments was to carry out a test with more stringent conditions by exposing the mild steel to corrosive TMI process water augmented with SRB (*D. orientis*), and without allowing the protective *B. brevis* 18–3 biofilm to form first, to see if the gramicidin-S-producing *B. brevis* 18–3 biofilm could still inhibit the SRB *D. orientis*. *P. polymyxa* 10401 was used as a biofilm-forming control.

In two different sets of experiments, no H_2S generation was observed in the reactors with a protective biofilm of *B. brevis* 18–3, although some black precipitate was found on the metal coupon surface for one of the two sets after 3 days, indicating less severe growth of *D. orientis* than in reactors with the biofilm control *P. polymyxa* 10401, which smelled of H_2S after 1–2 days and turned black after 3–4 days in both sets of experiments. Analysis of the impedance spectra suggested that corrosion rates increased continuously in the test with the biofilm control, but remained lower and constant in the test with a protective biofilm of *B. brevis* 18–3 (Fig. 4). The average $1/(R_p \cdot A)$ values for the two different sets of experiments (Fig. 4) showed that the *B. brevis* 18–3 biofilm was still effective in inhibiting the corrosion of mild steel caused by *D. orientis* even under such difficult conditions. Based on the INT values for the data in Fig. 4, a 5.6-fold decrease in relative corrosion rate (E_{rel}=82%) was obtained in the presence of *B. brevis* 18–3 (Table 1). Corrosion rates obtained from the mass-loss data support the protective effect revealed by the impedance data since the average corrosion rate was 21 (17) μm/year [7 (6) mg] for the protective biofilm and 79 (60) μm/year [26 (20) mg] for the biofilm control (Table 1). The corrosion potential E_{corr} for the biofilm control was 157 mV higher than that for the protective biofilm (Fig. 5).

Continuous reactor inhibition of *D. vulgaris* in TMI process water

The antibiotic-producing biofilms *P. polymyxa* 10401–pBE92-Bac, *P. polymyxa* 10401–pBE92-Probac, *B. brevis* Nagano, and *B. brevis* 18–3 were tested independently for the inhibition of the Gram-negative SRB *D. vulgaris* and subsequent reduction in the corrosion rate of mild steel in TMI process water. The protective biofilm was estab-

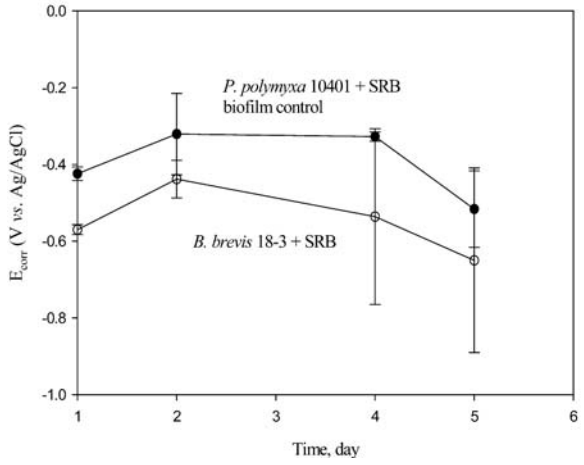


Fig. 5 Corrosion potential (E_{corr}) of mild steel 1010 in the presence of SRB (*D. orientis*) and *B. brevis* 18–3 (protective) or *P. polymyxa* 10401(control) biofilms in continuous reactors containing TMI water. Symbols and error bars as defined in Fig. 3

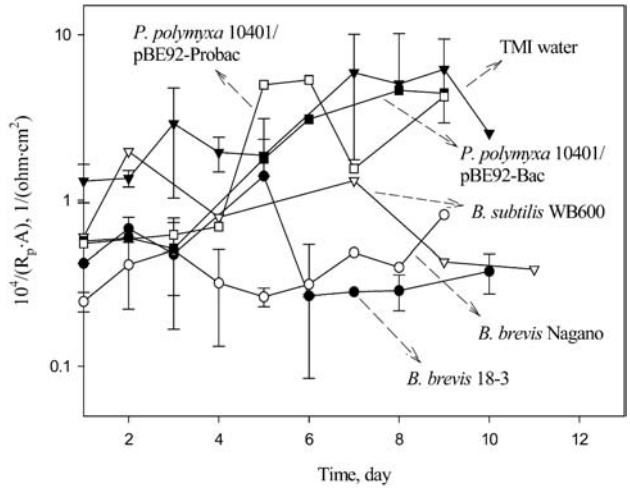


Fig. 6 Relative corrosion rates [$1/(R_p \cdot A)$] for mild steel 1010 in the presence of SRB (*D. vulgaris*) and the single biofilm of *B. brevis* 18–3 (● duplicate, protective), *B. brevis* Nagano (○ duplicate, protective), *P. polymyxa* 10401–pBE92-Probac (❑ single experiment, protective), *P. polymyxa* 10401–pBE92-Bac (■ single experiment, protective), or *B. subtilis* WB600 (▽ single experiment, control) in continuous reactors containing TMI service water. TMI service water without protective biofilm (▼ triplicate) was also used as a negative control. *Error bars* shown for the standard deviations for duplicate and triplicate experiments

lished first for 2 days, then challenged with SRB in the TMI feed. The impedance spectra showed that both the wild-type *B. brevis* Nagano biofilm and the mutant *B. brevis* 18–3 biofilm decreased the relative corrosion rate of mild steel. Table 1 lists the INT and corrosion rate values obtained from mass-loss data. Based on the INT values, corrosion damage was decreased 7.9- and 8.6-fold respectively (E_{rel}=87% and 88%) compared to the control

Table 1 Corrosion of mild steel 1010 in continuous experiments in the presence of sulfate-reducing bacteria (*SRB*). *INT* was the integral of $1/(R_p \cdot A)$ over time. Corrosion rate data (μm/year) were converted from experimental mass-loss data divided by the electrode area (30.58 cm^2, surface area of the tested metal exposed to the medium), the time of the experiments (year), and the density of mild steel (7.85 g/cm^3)

Protective biofilm	Medium	SRB	INT ($s/ohm \cdot cm^2$)	Corrosion rate (μm/year)	Impedance spectra ($1/(R_p \cdot A)$ vs. time)	E_{corr} (mV vs Ag/AgCl)
Bacillus brevis 18–3 (2 experiments)	Baars'	*Desulfosporosinus orientis*	ND	14 (2)	Not shown	–495 (10) (average of last 3 days, see Fig. 3)
P. polymyxa 10401 (biofilm control, 2 experiments)	Baars'	*D. orientis*	ND	36 (4)	Not shown	–393 (32) (average of last 3 days, see Fig. 3)
B. brevis 18–3 (2 experiments)	TMI	*D. orientis*	21 (5)	21 (17)	Fig. 4	–547 (98) (average of last 3 days, see Fig. 5)
P. polymyxa 10401 (biofilm control, 2 experiments)	TMI	*D. orientis*	115 (56)	79 (60)	Fig. 4	–390 (109) (average of last 3 days, see Fig. 5)
B. brevis 18–3 (2 experiments)	TMI	*Desulfovibrio vulgaris*	28.3 (0.4)	27 (5)	Fig. 6	–637 (92) (average of last 3 days, see Fig. 7)
B. brevis Nagano (1 of 2 experiments)	TMI	*D. vulgaris*	31	24	Fig. 6	–653 (42) (average of last 3 days, see Fig. 7)
P. polymyxa 10401 –pBE92-Bac (1 experiment)	TMI	*D. vulgaris*	156	NA	Fig. 6	–640 (187) (average of last 3 days, see Fig. 7)
P. polymyxa 10401 –pBE92-proBac (1 of 1 experiment)	TMI	*D. vulgaris*	164	NA	Fig. 6	–402 (201) (average of last 3 days, see Fig. 7)
TMI water without biofilm (2 of 3 experiments)	TMI	*D. vulgaris*	243 (62)	47 (28)	Fig. 6	–538 (127) (average of last 3 days, see Fig. 7)
B. subtilis WB600 (biofilm control) (1 experiment)	TMI	*D. vulgaris*	91	44.2	Fig. 6	–791 (2) (average of last 3 days, see Fig. 7)

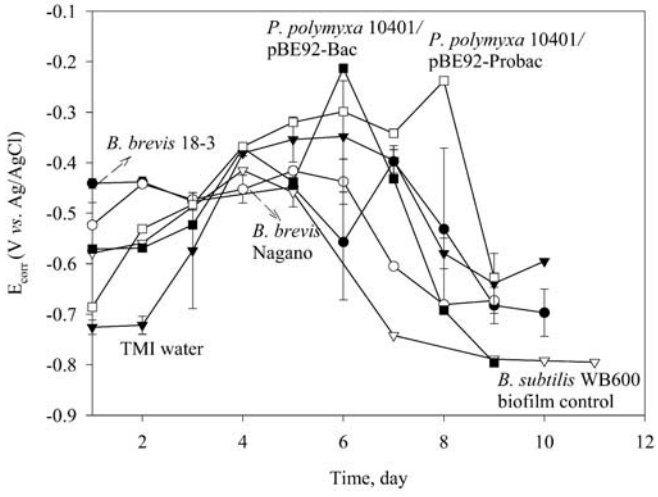


Fig. 7 Corrosion potential (E_{corr}) of mild steel 1010 in the presence of SRB (*D. vulgaris*) and the single biofilm of *B. brevis* 18–3, *B. brevis* Nagano, *P. polymyxa* 10401–pBE92-Probac, *P. polymyxa* 10401–pBE92-Bac, or *B. subtilis* WB600 in continuous reactors containing TMI service water. TMI service water without protective biofilm was also used as a negative control. Symbols and error bars as defined in Fig. 6

of TMI without a protective biofilm (Table 1 and Fig. 6). In the presence of *B. brevis* Nagano and *B. brevis* 18–3, the relative corrosion rates based on the INT data in Table 1 were decreased 2.9- and 3.2-fold, giving the relative *E*-values of 66% and 69% respectively, compared to the *B. subtilis* WB600 biofilm control (Table 1 and Fig. 6). Corrosion rates based on mass-loss data were 27 (5) μm/year [18 (3.5) mg] for *B. brevis* 18–3 and 24 μm/year (14 mg) for *B. brevis* Nagano, compared to 47 (28) μm/year [31 (18) mg] for the negative control (TMI water without a biofilm) and 44.2 μm/year (32 mg) for the biofilm control test (Table 1).

The corrosion potential E_{corr} (the average of the last 3 days of the continuous experiments) for the protective biofilm [−637 (92) mV for *B. brevis* 18–3 and −653 (42) mV for *B. brevis* Nagano] was more negative than that for negative control [–538 (127) mV for TMI water without biofilm], and a little more positive than that for biofilm control [−791 (2) mV for *B. subtilis* WB600]. These data suggest that a good biofilm-former like *B. subtilis* WB600 (as evidenced by very thick biofilm formed on the metal surface during the continuous experiments, picture not shown) can establish a barrier to decrease the oxygen concentration at the metal surface, resulting in a lower rate of the cathodic reaction and hence in a reduced corrosion rate (Table 1 and Fig. 7). Nevertheless, this biofilm did not produce an antimicrobial against SRB and was not able to afford additional protection to mild steel, unlike the biofilms of *B. brevis* 18–3 and *B. brevis* Nagano.

The color of the solutions in the reactors indicated that the SRB were inhibited by the *B. brevis* Nagano biofilm since the medium was yellowish and there was no hydrogen sulfide odor, nor was a black biofilm visible on the metal surface. Similar results were obtained with *B. brevis* 18–3; however, the medium was black, a very strong smell of H_2S was released from the air outlet of the reactors (which was then absorbed by a sodium hydroxide solution to avoid odors), and the metal surface was covered with a strongly-attached black layer in the control test of TMI water without a protective biofilm. Similar results were obtained with the *B. subtilis* WB600 biofilm control. The protection afforded by the protective biofilm for mild steel was also corroborated by SEM analysis. Fig. 2c and d shows the surface appearance of polished mild steel with magnification 1,000x after 10 days exposure to the TMI water augmented with *D. vulgaris*. The polishing grooves are still distinct on the surface of the sample protected by the *B. brevis* 18–3 biofilm (Fig. 2c), while they are not visible on the surface of the sample without that protection (Fig. 2d). Hence, the extent of corrosion was decreased when a *B. brevis* 18–3 biofilm was present.

Analysis of the impedance spectra suggests that the protective biofilms of *P. polymyxa* 10401–pBE92-Bac and *P. polymyxa* 10401–pBE92-Probac (1.5 ml each of the cultures was inoculated into the reactors) did not provide additional protection to mild steel compared to the control of TMI process water and a *B. subtilis* WB600 biofilm (Table 1 and Figs. 6, 7). Similar to the control experiments, the medium was black, a very strong smell of H_2S was released from the outlet of the reactors, and the metal surface was covered with a black iron sulfide film in the presence of the recombinant *P. polymyxa* 10401 biofilms.

Continuous reactor inhibition of *D. vulgaris* in TMI process water with the antimicrobial GGPST consortium

In this set of experiments, a mixture of the protective bacteria cultures (GGPST consortium) was added to the reactor, and TMI water containing SRB was fed into the reactor via a pump at the same time (the protective biofilm was not allowed to develop first). Compared to the *B. subtilis* WB600 biofilm control, the antimicrobial GGPST consortium biofilms delayed the growth of *D. vulgaris* for one more day, protected mild steel and decreased the corrosion rate 2- to 10-fold (E_{rel}=50–90%) for up to 8 days as revealed by impedance data (data not shown). The mass-loss data for the mild steel at the end of the 12-day experiment showed no difference between the protective biofilms [Δm 61 (18) mg or corrosion rate of 77 (23) μm/year] and the biofilm controls [Δm=67 (30) mg or corrosion rate of 85 (38) μm/year]. No significant difference in E_{corr} was observed between the protective biofilms and the control biofilm (*B. subtilis* WB600). In a short-term (5–6 days) simultaneous, continuous experiment using a consortium of *B. brevis* 18–3 and *B. brevis* Nagano to inhibit *D. vulgaris* in TMI water, the impedance analysis showed a 3- to 4-fold reduction in the corrosion rate during the last 2 days of the

experiment, and mass-loss measurements showed a 2-fold reduction for the protective biofilms compared to the *B. subtilis* WB600 biofilm control, suggesting that impedance data and mass-loss data support each other in the short term.

Discussion

EIS data, SEM analysis, mass-loss data, and visual examination clearly showed that mild steel was protected from the attack of the Gram-positive *D. orientis* and the Gram-negative *D. vulgaris* in TMI water by protective *Bacillus* biofilms that produce the cationic antimicrobial peptide gramicidin S. The data presented suggest that the inhibition of SRB is due to the interaction of antimicrobial peptides produced by the *Bacillus* biofilms with the SRB biofilm. The experiments with the supernatant of gramicidin-S-producing bacteria culture and purified antimicrobial gramicidin S (1–100 μg/ml) showed that gramicidin S decreased the viability of both *D. orientis* and *D. vulgaris* in culture suspensions by more than 90% in 1 h. The killing mechanism of Gram-negative bacteria by the cationic antimicrobial peptides has been shown to involve disruption of the outer and cytoplasmic membranes (Friedrich et al. 2000; Zhang et al. 2001).

The inhibition of the SRB was due to biofilm cells and not suspension cells since after 6–7 days in the continuous reactor experiments with TMI water, the suspension cells were washed out leaving only the biofilm cells. During this time, the impedance data clearly showed inhibition of the SRB for the biofilms producing gramicidin S. Previously, we also found that the reduction in corrosion was due to the biofilm cells and not the cells in suspension since killing the biofilm cells resulted in the loss of corrosion protection and since washing out the cells in suspension (so that only biofilm cells were present) did not decrease the corrosion protection (Jayaraman et al. 1997a). Also, previously we showed the protection effect was due to a *B. brevis* 18–3 biofilm and not suspension cells (Jayaraman et al. 1999c), and showed that *Bacillus* strains make biofilms on mild steel (Jayaraman et al. 1997b).

When the protective biofilm was established on the clean metal surface before exposure to *D. orientis* in Baars' medium or *D. vulgaris* in TMI water, no iron sulfide film formation occurred on the metal surface in the presence of the protective biofilm during the entire 6-day test period in experiments with *D. orientis* or during the 12-day test period with *D. vulgaris* experiments. This indicates that the biofilms protected the mild steel from attack by SRB. The protective biofilms covered the metal surface and thereby protected the mild steel from the invasion of corrosion-causing SRB, even though nutrients were being depleted in these experiments because it was not possible to add the TMI water continuously at the flow rates seen in the field. When the biofilm control (*B. subtilis* WB600) was established first, the attack by *D. vulgaris* caused less corrosion damage (mass loss was 44.2 μm/year or 32 mg) than when it was added simultaneously with the control biofilm in the experiments testing the inhibition ability of GGPST consortium [mass loss was 85 (38) μm/year or 67 (30) mg]. This suggests that simultaneous attack by SRB posed a tougher challenge on the corrosion-inhibition potential of gramicidin-S-producing bacteria. Under such conditions, the *B. brevis* 18–3 biofilm still showed the ability to protect the mild steel in TMI water, even when simultaneously challenged with *D. orientis*.

When examining the corrosion of mild steel exposed to *D. orientis* in Baars' medium, it is to be expected that the corrosion rate data obtained from mass-loss data in batch operation (the 21-day experiments with supernatants) would be lower than that in continuous operation (the 6-day continuous experiments). This is because the continuous flow of feed brought more oxygen to the metal surface and therefore more corrosion damage appeared compared to the stagnant and anaerobic environment in the batch operation. This protection effect by lowering the oxygen concentration at a metal surface was also shown with *B. subtilis* WB600 in corrosive TMI water. INT values and E_{corr} data suggested that a good biofilm-former like *B. subtilis* WB600 decreased the corrosion rate of mild steel compared to the negative control (TMI water without any biofilm). When an antimicrobial (gramicidin S) against SRB (*D. vulgaris*) was produced by *B. brevis* 18–3 or *B. brevis* Nagano, additional protection was provided.

Our study is the first to use in-situ antimicrobial-producing biofilms to reduce corrosion of mild steel in the presence of SRB in real-world TMI process water. The current system offers an alternative method to control MIC and may be applicable in the inhibition of other corrosion-causing bacteria.

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