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Inhibiting sulfate-reducing bacteria in biofilms on steel with antimicrobial peptides generated in situ

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Abstract In batch and continuous fermentations, the reduction in corrosion of SAE 1018 mild steel and 304 stainless steel caused by inhibition of the reference sulfate-reducing bacterium (SRB) *Desulfovibrio vulgaris* by a protective, antimicrobial-producing *Bacillus brevis* biofilm was investigated. The presence of *D. vulgaris* produced a thick black precipitate on mild steel and a higher corrosion rate in batch cultures than that seen in a mono-culture of non-antimicrobial-producing *Pseudomonas fragi* K upon the addition of SRB to the aerobic *P. fragi* K biofilm. In continuous reactors, the polarization resistance R_p decreased for stainless steel and increased for mild steel upon the addition of SRB to a *P. fragi* K biofilm. Addition of either 200 μg/ml ampicillin, chloramphenicol, or ammonium molybdate to batch and continuous reactors after SRB had colonized the metal was ineffective in killing SRB, as inferred from the lack of change in both R_p and the impedance spectra. However, when ampicillin was added prior to SRB colonization, the growth of SRB was completely inhibited on stainless steel in continuous reactors. Prior addition of ampicillin was only able to delay the growth of SRB on mild steel in continuous reactors. External addition of the purified peptide antimicrobial agent gramicidin S prior to the addition of SRB also inhibited the growth of SRB on stainless steel in continuous reactors, and the SRB were also inhibited on stainless steel in both batch and continuous reactors by producing gramicidin S in situ in a protective biofilm when the gramicidin-S-overproducing strain *Bacillus brevis* 18 was used.

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Introduction

Metal surfaces are rapidly colonized by aerobic bacteria present in the bulk liquid phase (Geesey 1990). The upper layers of this biofilm are aerobic while the regions near the metal surface can be anoxic because of the depletion of oxygen by the biofilm (Blenkinsopp and Costerton 1991). Sulfate-reducing bacteria (SRB) can colonize these anaerobic niches and thus are primarily responsible for corrosion even in an aerobic environment (Hamilton 1990).

SRB have been implicated in the deterioration of metals for pipelines and off-shore oil rigs (Hamilton 1983), cooling water-recirculation systems (Borenstein 1994), sewage treatment facilities and pipelines (Odom 1990), jet fuel tanks (Miller 1981), and power generation equipment. SRB can cause corrosion of a wide range of metals including low-grade carbon steels (Dubey et al. 1995), stainless steels (Benbouzid-Rollet et al. 1991), and copper alloys (Licina 1989). It has been estimated that corrosion damage due to SRB in the U.S. costs $4–6 billion/year (Beloglazov et al. 1991).

For closed systems like cooling towers and storage tanks, biocides are probably the commonest method of combating biocorrosion (Cheung and Beech 1996). Saleh et al. (1964) have reviewed the use of nearly 200 compounds that are bactericidal or bacteriostatic with SRB. Oxidizing biocides like chlorine, chloramines, and chlorinating compounds are used in freshwater systems (Boivin 1995). Chlorine compounds are the most practical biocides; however, their activity depends on the pH of the water and the amount of light and temperature (Keevil et al. 1990), and they are not very effective against biofilm bacteria (Boivin 1995). Non-oxidizing biocides, such as quartenary salts (Beloglazov et al. 1991), amine-type compounds, anthraquinones (Cooling III et al. 1996), and aldehydes (Boivin 1995), are more

stable and can be used in a variety of environments. However, the environmental impact and cost of adding large quantities of inorganic compounds to open systems must also be considered when such biocides are used.

Diffusional limitations imposed by the glycocalyx (Hoyle et al. 1990), phenotypical changes like the expression of the *algC* gene of *Pseudomonas aeruginosa* that occur in the biofilm (Costerton et al. 1995), and the effect of surface chemistry on the metabolic state of the biofilm (Keevil et al. 1990) may all serve to increase the resistance of biofilms to antimicrobials beyond that observed with planktonic bacteria (Brown and Gilbert 1993). SRB are inherently resistant to a wide range of antimicrobials (Saleh et al. 1964), and the harsh anaerobic environment (created by corrosion products) in which the SRB thrive also reduces antimicrobial efficiency (Cheung and Beech 1996). Once SRB are firmly established in their niche, it is commonly very difficult to eliminate them completely from the system without disassembling it (Boivin 1995). Given this difficulty of killing SRB in biofilms, and that Miller (1981) and Cord-Ruwisch et al. (1987) have observed that microbiological sterility in a natural system is unachievable, it seems reasonable to develop strategies to combat the colonization of SRB from within the biofilm itself rather than using high doses of biocides to inhibit them after they are established. Specifically, an aerobic, biofilm-forming bacterium could be genetically engineered to produce antimicrobials that would exclude SRB from colonizing the biofilm (Jayaraman et al. 1999).

In this study, a novel strategy is developed for inhibiting SRB colonization and anaerobic corrosion in biofilms on mild steel and stainless steel. The commonly used antibiotic ampicillin has been used as a reference antimicrobial to show that addition prior to SRB colonization can be a viable approach to reduce SRB-induced corrosion. The ten-amino acid cyclic peptide gramicidin S (Azuma et al. 1992) has been identified to inhibit SRB (Jayaraman et al. 1999) and was also added externally as a model peptide antimicrobial to demonstrate the feasibility of producing peptide antimicrobials in biofilms to inhibit corrosion of mild steel and stainless steel. Furthermore, a gramicidin-S-overproducing *Bacillus brevis* Nagano strain (Azuma et al. 1992; *B. brevis* 18) has been used to establish a biofilm that secretes gramicidin S and inhibits SRB on stainless steel.

Materials and methods

Bacterial strains, medium, and growth conditions

All aerobic bacteria were grown from single colonies in 10 ml modified Baar's medium (American Type Culture Collection medium 1249) at 30 °C and 250 rpm (series 25 shaker; New Brunswick Scientific, Edison, N.J.) and used as the inoculum for biofilm development. The aerobic *Bacillus* bacteria were obtained from ATCC as shown in Table 1 except for *Bacillus brevis* 18, which was obtained from Prof. A.L. Demain (Massachusetts Institute of Technology). *Pseudomonas fragi* K is a kanamycin-resistant transposon mutant of the spoiled-meat bacterium *P. fragi* ATCC 4973 (Jayaraman et al. 1997a), which is also naturally resistant to both ampicillin and gramicidin S at 100 μg/ml. *Desulfovibrio vulgaris* (ATCC 29579) was cultivated in 15-ml screwcap tubes containing 10 ml modified Baar's medium supplemented with 100 μl each of the oxygen scavengers 4% sodium sulfide and Oxyrase (Oxyrase Inc., Mansfeld, Ohio). Initial cultures were grown from −85 °C glycerol stocks; all subsequent cultures were grown with a 3% inoculum from the initial culture at 30 °C without shaking. *D. vulgaris* was routinely cultured in tightly closed screwcap tubes and exposed to oxygen in air without any difficulty in cultivation (as reported by Angell and White 1995). *D. vulgaris* was also cultured periodically in the presence of 0.1% ferrous ammonium sulfate, and the presence of sulfate reducers was confirmed by the detection of black iron sulfide in the culture tubes. The desulfoviridin assay (Postgate 1984) was also routinely performed with the detection of a pink color under UV light to confirm the presence of *D. vulgaris*. Gramicidin S was obtained from Sigma Chemical Company (St. Louis, Mo.), chloramphenicol from Fisher Scientific (Pittsburgh, PA), and ammonium molybdate from Aldrich Chemical Company (St. Louis, Mo.).

Metal coupon preparation

Mild steel SAE 1018 coupons for batch culture experiments (25.5 mm diameter and 1.2 mm thick) and SAE 1018 mild-steel and stainless-steel 304 plates for continuous culture experiments (7.5 × 7.5 cm squares, 1.2 mm thick) were cut from sheet stock and prepared as reported previously (Jayaraman et al. 1997a).

Batch culture corrosion experiments

Batch culture corrosion experiments were performed in 250-ml conical flasks at 30 °C without shaking as described previously (Jayaraman et al. 1997a). Mild-steel coupons (triplicates) exposed to *D. vulgaris* were cleaned by wiping the surface with 0.01% chromic acid followed by repeated washes in warm water; all other coupons were cleaned as described earlier (Jayaraman et al. 1997a). The specific mass loss (in mg/cm^2 for the total surface area of the coupon, 11.18 cm^2) was used as an indicator of the extent of corrosion (assumed uniform). The growth medium was replenished every 7 days and replaced (with appropriate antibiotics) by gentle addition along the walls of the flask. A 3% (v/v) SRB inoculum was added to the flasks after 3 days of aerobic biofilm development.

Continuous-culture corrosion experiments using EIS

Continuous reactors operating for 200–400 h (in at least duplicate experiments) were used to develop biofilms on metal surfaces, and electrochemical impedance spectroscopy (EIS) was used to obtain impedance data as described earlier (Jayaraman et al. 1997c). The open-circuit potential was measured as the potential between the metal specimen and the reference electrode (Ag/AgCl), and the polarization resistance (R_p) was determined as the d.c. limit of the impedance, using the ANALEIS software developed by Mansfeld et al. (1992). Continuous-culture corrosion rates were estimated from the experimental R_p on basis of the Stern-Geary equation $R_p = B/i_{corr}$, where B is a parameter depending on the Tafel slopes and i_{corr} is the corrosion current density, which can be converted into a corrosion rate by using Faraday's law (Mansfeld 1976).

A 1% (v/v) SRB inoculum (culture age 24–48 h) was added to the reactor after 3–5 days of aerobic biofilm development. On the basis of the minimum inhibitory concentrations available in the literature (Saleh et al. 1964) and also on data generated in this laboratory on the susceptibility of SRB in suspension cultures to various inorganics and antimicrobials (data not shown), ampicillin (200 μg/ml), chloramphenicol (200 μg/ml), both ampicillin (200 μg/ml) and chloramphenicol (100 μg/ml), and both ampicillin (200 μg/ml) and ammonium molybdate (200 μg/ml) were added to reactors (before or after SRB had colonized the metal) in an attempt to inhibit SRB in the biofilm while not inhibiting *P. fragi* K. All

antimicrobials were simultaneously added to the feed bottle and the reactor at appropriate concentrations.

Enumeration of viable SRB in biofilms

Aerobic biofilms were developed on 304 stainless steel coupons (25.5 mm diameter, 1.2 mm thick) in 250-ml conical flasks for 2 days with modified Baar's medium at 30 °C. A 1.0% (v/v) inoculum of *D. vulgaris* (A_{600} = 0.16–0.18) was added and allowed to colonize the biofilm for an additional 4 days. The metal coupons were carefully removed from the flasks and rinsed twice by immersion in distilled water to remove loosely attached cells. The biofilm was then scraped off with a sterile spatula and resuspended in 500 µl modified Baar's medium. Aerobic bacteria were determined by plate counts and viable SRB were enumerated by the three-tube MPN assay (Anonymous 1992).

Results

Batch and continuous corrosion with non-antimicrobial-producing *P. fragi* K and *D. vulgaris* on SAE 1018 mild steel

Mass loss from mild-steel SAE 1018 coupons in modified Baar's medium in the presence of *P. fragi* K and *D. vulgaris* was examined every 3–7 days for 32 days in stationary batch cultures at 30 °C (note a 3% SRB inoculum was used to pose a robust challenge to the aerobic biofilm and to facilitate SRB integration into the biofilm). Whenever *D. vulgaris* was present in the biofilm, the coupons were covered with a thick, black deposit and were difficult to clean. A dual culture of *P. fragi* K and *D. vulgaris* produced a 1.8-fold increase in corrosion after 21 days of exposure compared to a mono-culture of *P. fragi* K [these data were similar to those reported by Jack et al. (1992) and Gaylarde (1992)]; however, the corrosion observed in both cases was always lower than that observed with sterile modified Baar's medium (Table 1). The corrosion observed with a mono-culture of *D. vulgaris* on SAE 1018 steel was higher than that in sterile medium after 14 days (1.4-fold) and 21 days (2.5-fold, interpolated from Table 1). When ampicillin (100 µg/ml) was added to the flasks with *P. fragi* K before *D. vulgaris* was allowed to colonize the metal coupon, the mass loss observed was 29% (10 days) to 13% (3 weeks) less than that seen when ampicillin was added after SRB (Table 1). *P. fragi* K grew to saturation in overnight suspension cultures exposed to 100 µg/ml ampicillin or gramicidin S; therefore, it was not affected by adding these antimicrobials.

As indicated by the development of a black iron sulfide precipitate and an odor of hydrogen sulfide from the reactor outlet, the normally anaerobic *D. vulgaris* grew in continuous reactors as a mono-culture with an airflow rate of 200 ml/min to the headspace. Growth of *D. vulgaris* in continuous reactors increased R_p 90-fold after 72 h compared to sterile controls. Addition of 200 µg/ml ampicillin after 240 h of SRB growth did not change R_p, and the reactor remained black with the distinct odor of sulfide from the exhaust (Table 2).

Table 1 Corrosion loss of SAE 1018 steel in batch dual cultures of aerobic bacteria and representative sulfate-reducing bacteria (SRB). The order of antimicrobial and SRB addition to the fermentation is indicated by +

Bacterial strain (s)	Antimicrobial produced	Corrosion loss (mg/cm^2)						
		3 days	7 days	10 days	14 days	21 days	28 days	32 days
Sterile medium	–	–	0.54 ± 0.08	0.77 ± 0.11	–	1.03 ± 0.04	–	2.05 ± 0.11
P. fragi K	None	0.04 ± 0.01	–	0.19 ± 0.05	0.33 ± 0.05	0.38 ± 0.01		0.43
P. fragi K + SRB	None	0.04 ± 0.01		0.35 ± 0.01	0.52 ± 0.08	0.71 ± 0.08	–	0.86 ± 0.17
P. fragi K + SRB + Amp 100	None	0.04 ± 0.01	–	0.35 ± 0.01	0.42 ± 0.04	0.56 ± 0.08	–	0.65 ± 0.07
P. fragi K + Amp 100 + SRB	None	0.04 ± 0.01	–	0.25 ± 0.04	0.33 ± 0.04	0.49 ± 0.07		–
D. vulgaris ATCC 29579	None	0.10	0.19		1.23	–	3.83	–
B. subtilis ATCC 6633	Subtilin	0.13 ± 0.01	0.45 ± 0.08	–	0.57 ± 0.08	–	–	–
B. subtilis ATCC 6633 + SRB	Subtilin	0.13 ± 0.01	0.52 ± 0.03	–	0.81 ± 0.04	–	–	–
B. brevis ATCC 35690	Edeines	0.07 ± 0.01	0.16 ± 0.03	–	0.19 ± 0.01	–	–	–
B. brevis ATCC 35690 + SRB	Edeines	0.07 ± 0.01	0.23 ± 0.04	–	0.28 ± 0.03	–	–	–
B. brevis 18	Gramicidin S	0.09 ± 0.01	0.16 ± 0.02	–	0.28 ± 0.06	–	0.40 ± 0.06	–
B. brevis 18 + SRB	Gramicidin S	–	0.19 ± 0.02	–	0.30 ± 0.07	–	0.44 ± 0.06	

Table 2 Corrosion behavior of SAE 1018 steel in continuous reactors with dual cultures of aerobe and SRB after various methods of killing SRB. The order of antimicrobial and SRB addition to the fermentation is indicated by +. *EIS* electrochemical impedance spectroscopy R_p polarization resistance

Experiment	Antimicrobial added to kill SRB (μg/ml)	Time elapsed after antimicrobial addition (h)	Reactor characteristics	R_p, (Ω cm^2) before SRB addition (days)	R_p, (Ω cm^2) after antimicrobial addition (days)	R_p, (Ω cm^2) from mass loss data in Table 1 (days)	EIS spectra
D. vulgaris (1 of 2 experiments)	Ampicillin 200 added after 240 h of SRB growth	200	Reactor turned black and outlet had odor of sulfide. No changes observed after antimicrobial addition	3.58×10^3 (0)	3.24×10^5 (10)	5.7×10^3 (14)	Not shown
P. fragi K + SRB + ampicillin (1 of 2 experiments)	Ampicillin 200 added after 120 h of SRB growth	200	Reactor turned black and outlet had odor of sulfide. No changes observed after antimicrobial addition	4.52×10^4 (2)	1.35×10^5 (7)	3.76×10^4 (10)	Fig. 1
P. fragi K + ampicillin +SRB (1 of 3 experiments)	Ampicillin 100 added before SRB addition	100	Reactor did not turn black and outlet did not smell of sulfide	4.52×10^4 (2)	2.60×10^4 (9)	2.00×10^4 (10)	Fig. 1
P. fragi K + gramicidin S + SRB (1 of 1 experiment)	Gramicidin S 100 added before SRB addition	80	Reactor did not turn black, but outlet had odor of sulfide	4.52×10^4 (2)	3.89×10^4 (6)	Not calculated	Fig. 3
B. brevis 18 + SRB (1 of 2 experiments)	Gramicidin S (produced in situ before SRB addition)	48	Reactor did not turn black, but outlet had odor of sulfide	3.43×10^4 (3)	5.78×10^4 (8)	2.34×10^4 (14)	Fig. 3
B. brevis 35690 (1 of 2 experiments)	Edeines (produced in situ before SRB addition)	250	Reactor did not turn black, but outlet had odor of sulfide	3.75×10^4 (3)	Not calculated	Not calculated	Not shown

A combination of 200 μg/ml ampicillin and 200 μg/ml ammonium molybdate after 320 h cleared the reactor supernatant; however, the odor of sulfide was still detected, indicating that corrosion did not decrease and SRB activity was not inhibited.

The addition of *D. vulgaris* to a continuous *P. fragi* K reactor increased the R_p of mild steel 3-fold after 36 h and changed the frequency dependence of the phase angle; the reactor turned black and the odor of sulfide was detected from the reactor outlet (Table 2 and Fig. 1). Prior to the addition of *D. vulgaris*, the impedance attained a steady asymptotic value at low frequency (4.52×10^4 Ω cm^2); however, within 24 h of SRB addition, the reactor turned black, the odor of sulfide was detected, and the impedance no longer reached an asymptotic value at the lowest frequency (1.4×10^{-3} Hz). Addition of 200 μg/ml ampicillin (Table 2) and a combination of 100 μg/ml ampicillin and 25 μg/ml chloramphenicol after 120 h and 150 h of SRB growth (data not shown) also did not shift R_p to its value prior to SRB addition, indicating that there was no inhibition of SRB.

Fig. 1 Representative impedance spectra for continuous fermentations with SAE 1018 mild steel in modified Baar's medium with the purified antimicrobial ampicillin (*Amp*) added before and after sulfate-reducing bacteria (*SRB*) addition. Data are from a representative experiment (from a minimum of two independent experiments). The order of antimicrobial and SRB addition to the fermentation is indicated by +; $|Z|$ impedance modulus

Continuous corrosion rates with non-antimicrobial-producing *P. fragi* K and *D. vulgaris* on 304 stainless steel

No difference was observed between the impedance spectra for sterile Baar's medium and that with *P. fragi* K on 304 stainless steel after nearly 900 h of exposure. *D. vulgaris* did not grow as a mono-culture on 304 stainless steel, and the addition of *D. vulgaris* to a *P. fragi* K reactor changed the frequency dependence of the impedance at lower frequencies within 48 h (Fig. 2). The phase angle showed a minimum upon addition of SRB, indicating the appearance of a new time constant at very low frequencies (Fig. 2), and the maximum value of the phase angle decreased from 81° to 69°.

The changes of the impedance spectra were accompanied by the detection of the odor of sulfide from the reactor outlet, and the reactor also turned gray. Addition of 200 μg/ml ampicillin (Fig. 2), both 200 μg/ml ampicillin and 100 μg/ml chloramphenicol (Table 3), or both 200 μg/ml ampicillin and 200 μg/ml ammonium molybdate (data not shown) to a dual-culture reactor did not change the impedance spectra to the simple one-time-constant behavior observed prior to *D. vulgaris* addition (Fig. 2) or stop the production of hydrogen sulfide and iron sulfide, indicating that SRB had not been killed.

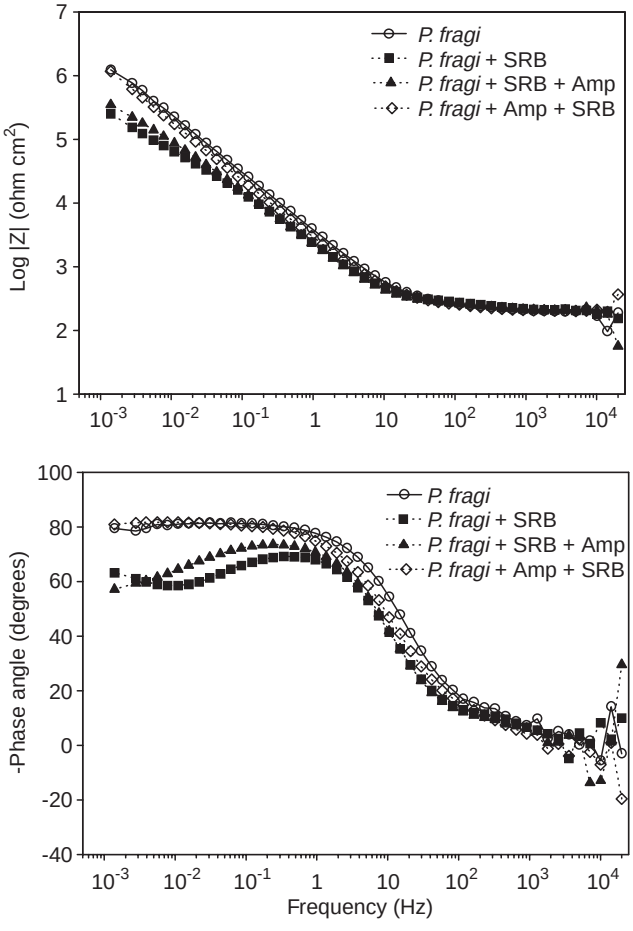

Fig. 2 Representative impedance spectra for continuous fermentations of 304 stainless steel in modified Baar's medium with the purified antimicrobial ampicillin added before and after SRB addition. Data are from a representative experiment (minimum of two independent experiments). The order of antimicrobial and SRB addition to the fermentation is indicated by +

Continuous corrosion rates with the biofilm exposed to purified SRB-inhibiting antimicrobials before addition of *D. vulgaris*

To determine whether antimicrobials are effective in inhibiting SRB when added prior to SRB colonization, non-antimicrobial-producing *P. fragi* K biofilms on SAE 1018 mild steel and 304 stainless steel were exposed to 100 μg/ml ampicillin or gramicidin S for 24 h before *D. vulgaris* was added. When *D. vulgaris* was added to mild-steel and stainless-steel reactors after the addition of ampicillin, no change in the impedance spectra and R_p was observed for up to 100 h (Figs. 1, 2; Tables 2, 3). No odor of sulfide was detected in the reactor outlet; hence, *D. vulgaris* was completely inhibited in the reactors by this antimicrobial. External addition of the cyclic decapeptide antimicrobial, gramicidin S, at 100 μg/ml was also completely effective in inhibiting the growth of *D. vulgaris* in the experiments on 304 stainless steel, as shown by the capacitive nature of the impedance spectra (Fig. 4 and Table 3); however, with mild steel, the reactor turned gray although there was no increase in R_p after 80 h of exposure to SRB (Fig. 3 and Table 2). Hence, the onset of *D. vulgaris*-induced corrosion of mild steel was later than that of a dual culture of *P. fragi* K and *D. vulgaris* without any gramicidin S present.

Batch and continuous corrosion rates with antimicrobial-producing-bacilli and *D. vulgaris* on SAE 1018 mild steel and 304 stainless steel

Batch corrosion studies of SAE 1018 steel coupons with *D. vulgaris* and antimicrobial-producing-*Bacillus* biofilms (based on their reported production of antimicrobial peptides, Table 1) demonstrated that all the bacilli were able to restrict the colonization of *D. vulgaris* for up to 1 week (demonstrated by the smaller 1.2- to 1.4-fold increase in corrosion, as compared to a larger 1.8-fold increase for *P. fragi* K in modified Baar's medium, Table 1, and also based on a lack of development of black color and sulfide odor). However, when the medium was replenished after 7 days, all the flasks except those with *B. brevis* 18 turned black, and iron sulfide was detected within 24 h (*B. brevis* 18 inhibited SRB for up to 28 days); the increase in corrosion rate with *D. vulgaris* in the presence of bacilli other than *B. brevis* 18 (1.2- to 1.5-fold increase) was comparable to that seen with *P. fragi* K and *D. vulgaris* (1.6-fold increase). However, the mass loss observed with *B. brevis* 18 and SRB was comparable to that with *P. fragi* K alone and

Table 3 Corrosion behavior of 304 stainless steel in continuous reactors with dual cultures of aerobe and SRB after various methods of killing SRB. The order of antimicrobial and SRB addition to the fermentation is indicated by +

Experiment	Antimicrobials added to kill SRB (μg/ml)	Time elapsed after antimicrobial addition (h)	Reactor characteristics	EIS spectra
P. fragi K + SRB + ampicillin (1 of 3 experiments)	Ampicillin 200 after 170 h and chloramphenicol 100 added after 400 h of SRB growth	490	Reactor turned gray and outlet had odor of sulfide upon SRB addition. No changes observed after antimicrobial addition	Fig. 2
P. fragi K + ampicillin + SRB (1 of 3 experiments)	Ampicillin 100 added before SRB addition	120	Reactor never turned gray and outlet did not have odor of sulfide upon SRB addition	Fig. 2
P. fragi K + gramicidin S + SRB (1 of 1 experiment)	Gramicidin S 100 added before SRB addition	130	Reactor never turned gray and outlet did not have odor of sulfide upon SRB addition	Fig. 4
B. brevis 18 + *D. vulgaris* (1 of 3 experiments)	Gramicidin S (produced in situ before SRB addition)	150	Reactor never turned gray and outlet had mild odor of sulfide upon SRB addition	Fig. 4
B. brevis (Nagano) + *D. vulgaris* (1 of 2 experiments)	Gramicidin S (produced in situ before SRB addition)	190	Reactor turned gray and outlet had odor of sulfide upon SRB addition	Not shown

nearly 2-fold better than with *P. fragi* K and SRB (Table 1). No odor of sulfide was detected throughout the experiment. The effectiveness of biofilm-generated gramicidin S in inhibiting the growth of SRB in batch cultures was also corroborated by the three-orders of magnitude less viable SRB being detected (by the three-tube MPN assay) in a *B. brevis* 18 biofilm on 304 stainless steel after 4 days of growth compared to a non-antimicrobial-producing *P. fragi* K biofilm (5.47×10^2/ml vs. 8.47×10^5/ml). These results indicate the potential of gramicidin S for killing SRB and demonstrate that the addition of antimicrobials prior to SRB colonization may be successful in inhibiting the growth of SRB.

Continuous-culture corrosion rates with *B. brevis* 18, a gramicidin-S-hyper-producing strain (Azuma et al. 1992) were obtained in the presence of *D. vulgaris* on SAE 1018 mild steel (Fig. 3, Table 1); the increase in R_p observed upon addition of *D. vulgaris* to *P. fragi* K on mild steel was delayed by 24 h. Eventually, SRB seem to

Fig. 3 Representative impedance spectra for continuous fermentations of SAE 1018 mild steel in modified Baar's medium with the purified antimicrobial gramicidin S (*GS*) added before SRB addition and gramicidin S generated in situ by the recombinant biofilm. Data are from a representative experiment (minimum of two independent experiments). The order of antimicrobial and SRB addition to the fermentation is indicated by the +

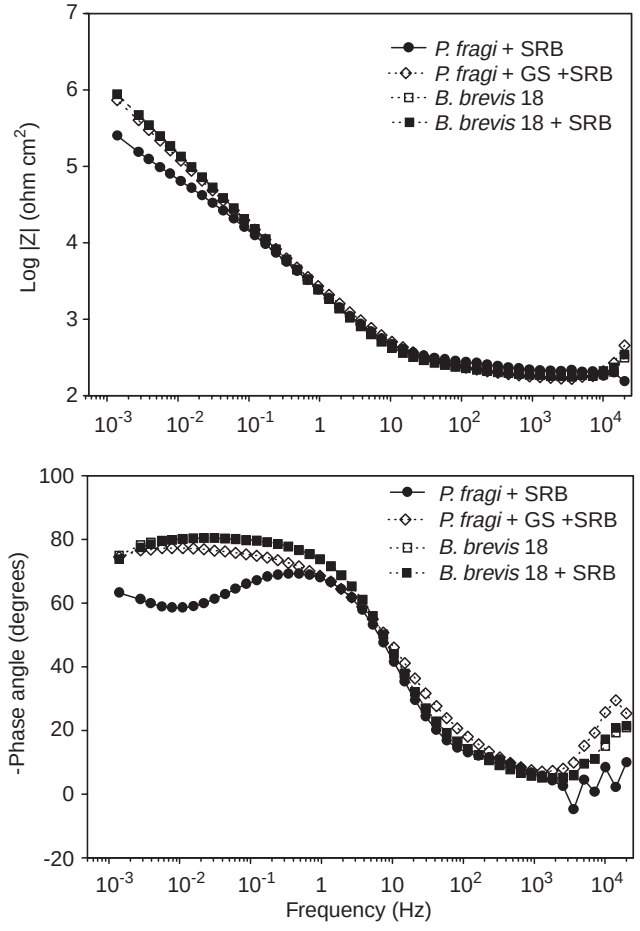

Fig. 4 Representative impedance spectra for continuous fermentations of 304 stainless steel in modified Baar's medium with the purified antimicrobial gramicidin S added before SRB addition and gramicidin S generated in situ by the recombinant biofilm. Data are from a representative experiment (minimum of two independent experiments). The order of antimicrobial and SRB addition to the fermentation is indicated by +

have colonized the biofilm, as shown by the odor of hydrogen sulfide from the reactor outlet; however, R_p remained constant at $5.78 \times 10^4\ \Omega\ cm^2$ as opposed to $3.43 \times 10^4\ \Omega\ cm^2$ before SRB addition.

Figure 4 and Table 3 show that the addition of *D. vulgaris* to a *B. brevis* 18 biofilm on type 304 stainless steel did not decrease R_p after 120 h, even though the smell of sulfide was detected in the reactor outlet 48 h after the addition of *D. vulgaris*. Therefore, the gramicidin-S-producing *B. brevis* 18 seems to be capable of inhibiting the colonization of SRB on 304 stainless steel, while it can probably only delay the growth of SRB on SAE 1018 mild steel.

Discussion

D. vulgaris was chosen as the representative sulfate-reducing bacterium to study the effectiveness of antimicrobials produced in situ in inhibiting anaerobic corrosion, as it has been reported to accelerate corrosion (Gaylarde 1992) and withstand oxygen stress (Hardy and Hamilton 1981). *D. vulgaris* showed remarkable resilience in growing as a mono-culture in stationary batch cultures and continuous reactors with an oxygen-saturated headspace. The growth conditions for *D. vulgaris* in this study were very similar to those used by Gaylarde (1992) as well as by Hamilton and Lee (1995) and have been termed most aggressive for corrosion (small amount of oxygen present).

Electrical impedance spectroscopy of mild steel and stainless steel was used to indicate the presence of SRB continuously without disturbing the biofilm and to characterize the corrosion behavior observed in continuous cultures with these metals; note that previous results have shown that the impedance data generated in these reactors are relevant for the biofilm, not the supernatant bacteria (Jayaraman et al. 1997c). Simple one-time-constant behavior was observed with *P. fragi* K and *P. fragi* K + ampicillin + SRB (Fig. 1) on mild steel, which is typical for uniform corrosion in neutral media (Mansfeld and Lorenz 1991), and the R_p and capacitance values (C) obtained for these two experiments were similar (Table 2). For *P. fragi* K + SRB, the dependence of the phase angle ϕ on frequency at the lowest frequencies suggests the occurrence of a new time constant, which could be due to pitting, while the symmetrical frequency dependence of ϕ for *P. fragi* K + SRB + ampicillin and the shift of the entire impedance curve compared to that for *P. fragi* K could be due to a higher R_p (Fig. 1). Similar capacitance values were indicated in the spectra for *P. fragi* K + SRB and *P. fragi* K + SRB + ampicillin on mild steel; however, it was not possible to fit these spectra to a simple equivalent circuit and obtain quantitative values of R_p and C. The impedance spectra for *B. brevis* 18, *B. brevis* 18 + SRB, and *P. fragi* K + gramicidin S + SRB on mild steel exhibited the frequency dependence usually observed for uniform corrosion, and R_p could be determined as the d.c. limit ($\phi = 0°$) of the impedance modulus $|Z|$ (Fig. 3).

Stainless-steel samples exposed to reactors with sterile medium did not reach a steady low-frequency impedance value. The stainless-steel spectra for *P. fragi* K and *P. fragi* K + ampicillin + SRB were capacitive with high R_p values close to $2 \times 10^7\ \Omega\ cm^2$ and capacitance values between 100 $\mu F/cm^2$ and 200 $\mu F/cm^2$, indicating uniform corrosion typical of stainless steel in neutral media (Mansfeld and Lorenz 1991) (Fig. 2). While the extent of changes in corrosion rates for all exposure conditions cannot be accurately determined by fitting the experimental data to appropriate equivalent circuits, it appears the corrosion rate of 304 stainless steel was reduced by approximately 50-fold by adding ampicillin or gramicidin S prior to SRB and by producing gramicidin S in situ using *B. brevis* 18 (approximate R_p values of $1.9 \times 10^7\ \Omega\ cm^2$, $1.44 \times 10^7\ \Omega\ cm^2$, $1.69 \times 10^7\ \Omega\ cm^2$, and $4.6 \times 10^5\ \Omega\ cm^2$ for *P. fragi* + ampicillin + SRB, *P. fragi* + gramicidin S + SRB, *B. bre-*

vis 18 + SRB, and *P. fragi* + SRB + ampicillin respectively). Further, the absence of changes in the impedance spectra (before and after SRB addition) demonstrates inhibition of SRB on stainless steel when purified antimicrobials were present prior to the addition of SRB or when gramicidin S was generated by the biofilm itself (Fig. 4).

Ampicillin and chloramphenicol are known to inhibit suspension cultures of *D. vulgaris* at 1 μg/ml and 3 μg/ml respectively (Odom and Singleton Jr 1993). Since biofilms are known to be 10–1000 times more resistant to biocides (Cheung and Beech 1996), up to 200 μg/ml for both antibiotics was used in this study. However, when added after SRB had colonized the metal surface, these additives did not stop further production of sulfide or decrease the corrosion rates of the two types of steel. This is consistent with the observations of Franklin et al. (1991) who observed that SRB may be able to survive exposure to halogen biocides for at least 26 h, and that of Franklin et al. (1989), who reported a 3- to 4-orders-of-magnitude decrease in a biofilm population with biocide addition, but noted that the population reached its pretreatment density within 24-h of stopping the biocide dose. When ampicillin-containing medium was discontinued for the mild steel reactors in this study, SRB-induced corrosion was evident within 36 h.

Since biofilm formation on surfaces exposed to natural environments is inevitable (Iverson 1987; Miller 1981), it is reasonable to use biofilm-forming bacteria to exclude undesirable bacteria. It is feasible to clone peptide antibiotics that are small (10–35 amino acids) into bacteria (rather than conventional antibiotics where large operons or pathways need to be cloned for the expression of a single antibiotic). Since *Bacillus* sp. are known to form biofilms (Borenstein 1994) as well as secrete efficiently antimicrobial peptides that have a wide range of antimicrobial activity, like gramicidin S (Azuma et al. 1992) and polymyxin B (Fujita-Ichikawa and Tochikubo 1993), they can be potentially used to produce antimicrobials targeted against specific bacteria (Jayaraman et al. 1999). Since biofilms can form rapidly on exposed surfaces (Costerton et al. 1995), this system can exclude other bacterial species from the biofilm. Such a system also has the added advantage of being able to reduce the extent of generalized corrosion caused by oxygen by as much as 40-fold, as has been shown by Jayaraman et al. for SAE 1018 mild steel (1997b, c). Thus, production of antimicrobial peptides within a protective biofilm to inhibit the growth of SRB is an attractive alternative to the use of high biocide concentrations. In addition, the paradigm discussed here might be appropriate for medical applications; for example, inhibiting the colonization of dental implants by deleterious bacteria which cause infection and subsequent implant failure (Mellonig et al. 1995).

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