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The Corrosion Effects of Sulfate- and Ferric-Reducing Bacterial Consortia on Steel

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Fourteen thermotolerant and thermophilic bacterial isolates from a hot spring in Guanajuato State, Mexico, were tested for their ability to induce the corrosion of carbon steel in monocultures and, in selected cases, in mixed cultures and co-culture with a sulfate-reducing strain, SRB-M. Characterization by 16S rDNA showed that three of the thermophilic isolates (G9a, G9c, and G11) belong to the genus Bacillus, *one (G2) showed homology with* Bacillus *as well as* Geobacillus, *and the SRB-M strain is closely related to* Desulfotomaculum *sp. Ten of the fourteen thermophilic and thermotolerant isolates promoted significantly more corrosion than the sterile controls. Under microaerobic batch-culture conditions at 55°C, SRB-M in monoculture did not show a corrosive effect measured as weight loss; in a mixed culture with G2, G9a, and G11, however, the final corrosion was enhanced 3.6 times when compared to sterile controls. Co-cultures of "G" isolates with SRB-M showed various effects on the final corrosion, ranging from 1.4 to −1.2 times the control rates. Interestingly, G2, the only isolate able to reduce ferric ion, had the largest effect on microbial induced corrosion in mixed culture. Two different models of thermophilic bacterial communities, reconstructed with ferric- and sulfate-reducing isolates, had corrosion rates 4.8 and 5.0 times higher than the sterile controls. Our data show that bacterial strains with hydrogenase activity are not necessarily corrosive and that corrosion induction can be modified substantially by the metabolic background provided by the larger bacterial community, especially its ferric-reducing members.*

Keywords bacterial consortia, ferric-reducing bacteria, steel corrosion, sulfate-reducing bacteria, thermophilic bacteria

Introduction

Bacterial corrosion is a phenomenon that affects a wide range of industries and causes very considerable economic losses (Costello 1969; Hamilton 1994). Most of the studies

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of microbiologically induced corrosion (MIC) under anaerobic conditions have been conducted using sulfate-reducing bacteria (SRB). These bacteria are recognized as promoters of the cathodic depolarization process in steel (Hamilton 1994), which includes the consumption of the hydrogen, formed by reduction of protons by electrons, from the metal's surface (von Wolzogen and Van der Vlugt 1934). The hydrogen forms a film that prevents further reductions of protons, producing electrostatic isolation (pasivation). Consumption of the hydrogen by SRB breaks this balance. S^{2-} produced by SRB is also involved. Ferrous sulfide, formed by the reaction of biogenic sulfide ions with the metal surface, produces an adhesive film. According to King and Miller (1971), this mineral film acts as a cathode for hydrogen evolution, but it can also have a protective, pasivation effect (King et al. 1973; 1976) The removal of the protective film by bacteria may result in higher rates of corrosion (Little et al. 1998; Obuekwe et al. 1981a, 1981b, 1981c). Little et al. (1998) suggested that ferrous ions from bacterial ferric reduction prevent formation of a protective sulfide layer.

In natural conditions, sulfate- and ferric-reducing bacteria may coexist. The SRB are present in sediments and in biofilms with heterogeneous bacterial communities with various ecological niches. The characteristics of the members of the population modifies the metabolism of the community, creating new ecological niches. Anaerobic niches may exist within a biofilm, with aerobic interfaces (Llobet-Brossa et al. 1998; Okabe et al. 1999).

In high-temperature natural environments, the reactions of chemical corrosion are accelerated. These environments are characterized by considerable diversity of microorganisms (Madigan et al. 1997), many of which may have corrosive effects (Torres-Sánchez et al. 1997). With the aim of gaining greater understanding of these processes, thermophilic bacteria were isolated to test their effects, in pure culture and in reconstructed sulfate- and ferric-reducing consortia, on corrosion of carbon steel.

Materials and Methods

Isolation of Bacteria and Culture Conditions

Thermophilic bacteria were isolated from a hot spring "Cerrito de Agua Caliente" in the State of Guanajuato, 20 km west of Irapuato City, Mexico (20°37′ N, 101°35′ W). Two-gram samples of mud, in situ temperature 55°C and pH 8.5, were transported to the laboratory at CINVESTAV under anoxic conditions. Bottles of 150 mL capacity containing 100 mL of culture medium V1 [g/L: KNO_3, 2.0; KH_2PO_4, 2.0; Na_2CO_3 1.0; Na_2SO_4, 0.5; NH_4Cl, 1.0; $MgSO_4{\cdot}7H_2O$, 0.8; yeast extract, 1.0; glucose, 3.0; micronutrient solution for *Thiobacillus denitrificans* reported in Atlas and Parks (1993), 1 mL] were inoculated with the 2-g aliquots of mud. The bottles were incubated under aerobic or anaerobic conditions at 55°C and pH 8.0 for 72 h to stimulate growth. Subculturing of dilutions (10^{-4}–10^{-6}) resulted in isolation of cultures that were examined for purity by streaking them onto V1 agar (2%). Isolated colonies with consistent macroscopic and microscopic morphology, for at least three successive rounds of culturing, were selected for further study.

The bacterial isolates were cultivated on V1 agar plates without yeast extract to test for growth-factor dependence. Slime-forming capacity was examined by formation of a slime thread when the colony was touched with a microbiologic loop. The isolates were cultivated under microaerobic (CO_2 5–12% + O_2 5–15%) and anaerobic conditions (CO_2 4–7% + H_2, redox potential of −200 mV approximately). The atmospheres were generated by the Gaspac system (Becton Dickinson). Under anaerobic conditions, V1 agar plates were used, with and without KNO_3, to test growth dependence on NO_3^-. Sulfate-reducing capacity was tested in culture medium V9 (see later) under anaerobic conditions.

A thermophilic sulfate-reducing isolate, SRB-M, previously isolated from "Los Azufres," Michoacán State, Mexico, was maintained by subculturing in medium D2 (see below). Sulfate-reducing bacteria were kept in closed tubes of 25-mm diameter and 70-mL volume containing 35 mL of culture medium.

Hydrogen Uptake

Vacutainer tubes (10 mL) were injected with 6 mL of culture medium V9 with or without lactic acid or sodium acetate, or both. Then, H_2 or N_2 (chromatographic grade) was added with a syringe at atmospheric final pressure. The tubes were inoculated with 200 μL of 4-day-old SRB-M cultures and incubated at 55°C for 10 days. Tubes showing a black precipitate of FeS, or other sulfides in the culture medium, were considered positive for hydrogen uptake.

Ferric-Reducing Capacity

Tubes with V10 culture medium (g/L: Na_3PO_4, 0.50; Na_2CO_3, 0.25; Na_2SO_4, 0.10; $MgSO_4$, 0.20; KCl, 0.05; yeast extract, 0.10; glucose, 0.20; micronutrients solution, 1 mL; pH 8) were inoculated with the bacterial isolates, as follows. The medium had been deoxygenated in an anaerobic box by bubbling with chromatographic grade N_2. To maintain anaerobic conditions, 3 mL of mineral oil were added to each tube before sterilization. After sterilization, the culture medium was amended with sterile anoxic ferric citrate at 250 μM final concentration and then inoculated. The cultures were incubated for 36 h at 55°C. The concentration of Fe^{2+} was determined before and after incubation in each culture as well as in sterile uninoculated controls.

Analytical Techniques

Concentration of Fe^{2+} in the cultures was determined by the ferrozine assay as previously described (Sørensen 1982), making the appropriate dilutions. Dissolved sulfide content was measured in culture media by the methylene blue method (Cline 1969) with a simple modification: in order to eliminate turbidity interference due to bacterial growth, the samples were centrifuged and the supernatant was assayed.

Total cell numbers were determined by direct counting in a Petroff-Hauser chamber. Viable counts on D2 agar plates were made by serial dilution.

Molecular Analysis

Amplification, cloning, and sequence analysis of 16S rDNA were carried out as follows. Bacterial DNA was isolated using the Genomic Prep TM kit (Amersham Pharmacia Biotech Inc.) for cells and tissues according to manufacturer's instructions. In other cases, overnight cultures were centrifuged (10^4 rpm 10 min) and decanted, the pellet and residual culture medium were frozen at −80°C. Then, the pellet was defrosted and 5 μL suspended in 100 μL of 0.1% Tween 20. This suspension was used as the DNA template.

Amplification of ribosomal DNA was carried out by PCR, according to manufacturer's instructions (Boehringer Mannheim). Five μL of template DNA were mixed with the other reagents in a final volume of 100 μL. The primers fD1 (5′ CCGAATTCGTCGACAACA GAGTTTGATCCTGGCTCAG 3′) and rD1 (5′ CCCGGGATCCAAGCTTAAGGAGGTG ATCCAGCC 3′) useful for amplification of most of the eubacterial 16S ribosomal DNA

were used (Weisburg et al. 1991). Polymerase chain reactions were performed as follows: 94°C for 3 min, 35 cycles of 94°C, 57°C and 72°C for 1 min, and 72°C for 3 min.

Cloning of the 16S ribosomal DNA fragments was carried out using Boehringer Mannheim's kit for cloning PCR products. The cloning vector pMOS*Blue* with a restriction site *Sma*I was used according to the manufacturer's instructions, starting either from the PCR product from the reaction cocktail, or from the corresponding purified band from the agarose gel. The plasmid was introduced into *E. coli* DH5α by transformation. Transformants were selected using ampicillin and tetracyline for plasmid recombinant clones, and the lacZ interruption was used to select clones with the amplified 16S rDNA fragment according to the manufacturer's instructions. Screening for recombinants was carried out by isolating the plasmid by alkaline lysis according to Sambrook et al. (1989), digesting with *Sma*I and looking for a band of the same molecular weight as the PCR product used for cloning in agarose 1.5% gel. Plasmids with the 16S fragment were used for sequencing. The nucleotide sequence of the cloned fragment was determined with an ALF DNA sequencer (Pharmacia Biotech, Uppsala Sweden). Sequencing was achieved with primers fD1 and rD1 described above. Amplified sequences were compared against the NCBI GenBank (www.ncbi.nlm.nih.gov/blast) using the BLAST program.

Sequence-alignment and diversity dendrograms were constructed through the MegAlign 4.0 program of DNAstar. The aligned sequences were checked and manually cut to reduce errors before they were further used for diversity dendrogram construction by performing inference phylogenetic analyses (Clustal method).

Corrosion Measurements

High-carbon steel (1% C) coupons with an average area of 6.82 cm^2, or carbon steel (0.015–0.020% C) coupons with an average area of 12.9 cm^2 were used. The individual areas of the coupons were estimated by their density, weight, and geometry. The high-carbon steel coupons were cleaned by shaking in acetone for 30 min. Then they were brushed with a tooth-brush, using a modification of the method of Haruta et al. (1991), and weighed on an analytical scale. The carbon steel coupons were cleaned using a slight modification of the method of Bryant et al. (1991), subjected to ultrasonication in citric acid (5% w/v) for 5 min, and then rinsed in distilled water for 1 min. The coupons were flamed and weighed, then placed in closed culture tubes with 35 mL of head space and 35 mL of salt-rich culture medium V9 (g/L): K_2HPO_4, 1.0; Na_2CO_3, 1.0; Na_2SO_4, 0.5; $MgSO_4$ $7H_2O$, 2.0; yeast extract, 0.2; sodium acetate, 1.0; lactic acid, 85% v/v, 3 mL; pH 8.0); or modified organic rich *Desulfovibrio* (Atlas and Parks 1993) culture medium D2 (g/L): $MgSO_4.7H_2O$, 1.50; $Fe(NH_4)_2(SO_4)_2$, 0.10; glucose, 5.00; casein peptone, 5.00; meat extract, 3.00; yeast extract, 0.20; pH 8); or isolation culture medium V1. The tubes were inoculated individually with the bacterial isolates. In all of the experiments, not inoculated tubes were included as sterile controls. Tubes were incubated at 55°C for various periods according to the aims of each experiment, after which the coupons were removed and cleaned as previously described, and then weighed. Corrosion was measured as weight loss (Bryant et al. 1991). Microbially influenced corrosion (MIC) factor was defined as: (steel corrosion by bacterial culture) ÷ (steel corrosion by sterile control). Corrosion rates were calculated as linear regressions of weight loss against time.

To determine corrosion rates by SRB in our system, experiments were carried out using SRB-M cultures in V9 medium with various incubation times, with or without lactic acid as an energy source. Weight losses of high-carbon steel coupons placed in the medium cultures were monitored after various incubation times.

Corrosion Experiments with Reconstructed Thermophilic Communities

Two model thermophilic bacterial communities were experimentally constructed using isolates with diverse phenotypic characteristics. Bacteria from potentially different ecological niches were included. Model bacterial community I was constructed with 0.5 mL of overnight cultures of the following isolates: G11 (strictly aerobic, growth-factor requiring), G9a (anaerobic, fermentative, slime forming), G2 (anaerobic, ferric reducing), G8a (anaerobic, nitrate reducing), and SRB-M (anaerobic, sulfate reducing). None of these isolates was corrosive in monoculture in the salt-rich V1 culture medium, although all (with exception of G2) showed corrosive effects in the organic-rich D2. Model bacterial community II was constructed to test the role of bacteria lacking sulfate- and ferric-reducing capabilities. G2 and SRB-M (present in model I) were employed plus G8b and G9c. These latter isolates were selected because they showed induction of corrosion in monocultures in media V1 and D2, and because they had ecological niches similar to those of G8a and G9a, respectively.

Results

Bacterial Isolation

Fourteen bacterial isolates (G1–G13) were obtained from the collected "Cerrito de Agua Caliente" mud. Ten were considered thermophiles, since they did not grow at room temperature ($25^{\circ}C \pm 5^{\circ}C$), the rest were thermotolerants (isolates G9a, G9b, G9c, and G9H_2). All isolates were able to grow under aerobic conditions, G1a, G1CO_2, G6, and G11 were unable to grow anaerobically. Isolates G8a and G8b were able to grow under anaerobic conditions, only in the presence of nitrate. Isolate G2 had ferric-ion-reducing ability. Most of the isolates were considered to be fermentative since they were able to grow anaerobically without an inorganic electron acceptor; no sulfate-reducing bacteria were isolated from the Cerrito de Agua Caliente samples. Some of the isolates could not be cultivated in defined media requiring yeast extract for growth. This behavior was interpreted as dependence on nutritional factors from other strains in the natural bacterial community.

Five isolates showed slime-forming ability. This agglutinating potential is considered to be an important factor for reconstruction of model bacterial communities.

Hydrogen Uptake

Isolate SRB-M was cultivated in Vacutainer tubes in an atmosphere of N_2 or H_2. It was able to grow with lactic acid, acetate, or H_2 as the sole energy source, and, in the presence of H_2, it was able to grow with CO_2 as the sole carbon source.

Molecular Examination of Bacterial Isolates

Isolates SRB-M, G2, G11, G9a, and G9c, members of a reconstructed thermophilic bacterial community (see later), were examined by 16S rDNA sequence analysis. The average length of the rDNA sequences obtained from the 5′ and 3′ ends was 470 bp. These sequences were compared with the NCBI GenBank in searching for homologies. All of the isolates belonged to the *Bacillus/Clostridium* group of the Firmicutes. Sequences from G9a and G9c showed homology with sequences from *Bacillus licheniformis*, but G9a was closer (98% and 98%) than G9c (94% and 97%). G9a and G9c sequences showed 92 and 97% homology when they were compared. Isolate G11 showed homology with sequences from *Bacillus* sp. TGS537 (98 and 98%). Isolate G2 was related to *Bacillus thermoleovorans* (98% and 98% homology), and to *Geobacillus subterraneus* strain K (98 and 96%). The sequences

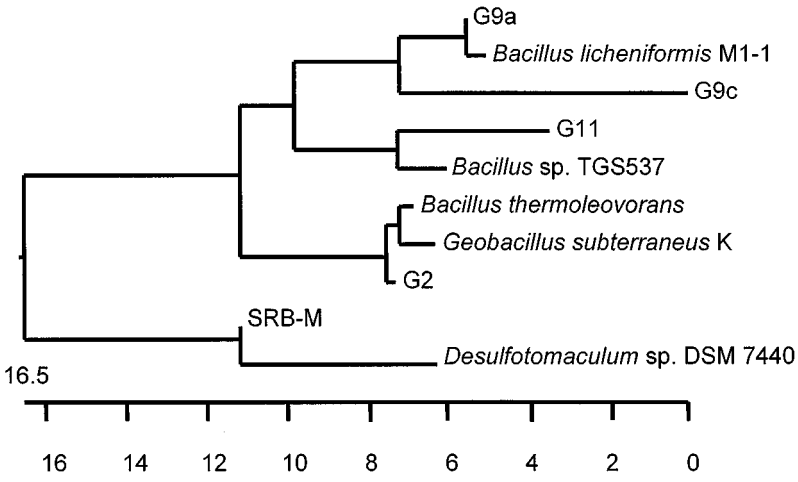

FIGURE 1 Diversity dendogram developed from the 16S rDNA 3′ end sequence (356 bases average) of G2, G9a, G9c, G11, and SRB-M (this work) and from the sequences of *Bacillus licheniformis* strain M1-1(AB039328), *Bacillus* sp. strain TGS537(AB020196), *Geobacillus subterraneus* strain K (AF276307), and *Desulfotomaculum* sp. strain DSM 7440 (DS16S7440) from GenBank (accession numbers in parenthesis).

from SRB-M showed homologies (94% and 96%) with sequences from *Desulfotomaculum* sp. DSM 7440.

Diversity dendograms showed similar branching patterns for our isolates, whether constructed from the 5′ (not shown) or 3′ (Figure 1) ends G9a and G9c were found to be closely related to G11 in a cluster with *B. licheniformis* and *Bacillus* sp TGS537. G2 was found to be more closely related to a *Bacillus-Geobacillus* cluster, therefore, its identification as a species of *Bacillus* or *Geobacillus* using a fraction of its 16S rDNA is not yet clear. SRB-M belonged to a separate *Bacillus* separacluster, close to *Desulfotomaculum*.

Corrosion Experiments

Monocultures of the bacterial isolates were tested for their ability to corrode carbon-steel coupons by incubating for 15 days in either the V1 or D2 medium. The weight-loss averages of at least four replicates were determined. Inoculation in the rich-salts V1 medium of most of the bacterial isolates did not result in corrosion values statistically different from those of the sterile controls. Isolates G9c and G8b, however, showed statistically significant ($\alpha \leq 0.05$) MIC factors of 1.65 and 1.38, respectively. Isolate G9c did not affect the pH of the medium, indicating that acidification was not a necessary component of corrosion. Cultures of isolates G4, G7, G2, and G9a showed MIC factors of 0.35 to 0.54 (a protective effect), statistically different to those of the sterile controls. Culture medium V1 seemed to be advantageous because of its buffering capability. Although corrosion rates in the sterile culture medium were high (average of 118 mg dm^{-2} in 15 days), effects in the G series of isolates were clearly observed.

When the same monocultures were evaluated for corrosion using rich organic medium D2, very different results were obtained (Table 1). A clear negative relationship between final carbon steel corrosion and final pH (coefficient of determination $r^2 = 0.96$) was obtained if G9c and G8b were omitted from the correlation analysis. Isolates G4, G6, G12, and G2 showed a final carbon steel corrosion statistically similar to those of sterile controls, whereas, in monoculture, the other isolates promoted carbon steel corrosion.

TABLE 1 Final corrosion of carbon steel coupons incubated in cultures of thermophilic isolates[a]

Isolates[b]	Final corrosion (mg dm^{-2})	Culture medium pH
G7	117[c] ± 8[d] A[e]	4.73[c] ± 0.23[d] A[e]
G10	111 ± 10 A B	4.77 ± 0.39 A
$G1CO_2$	111 ± 11 A B	4.84 ± 0.32 A
G11	108 ± 14 A B C	5.16 ± 0.51 A B
G1a	101 ± 7 A B C D	5.09 ± 0.59 A B
G8b	99 ± 6 B C D	6.21 ± 0.06 C
G9a	92 ± 9 C D	5.50 ± 0.31 A B C
G13	88 ± 9 D	5.46 ± 0.12 A B C
$G9H_2$	84 ± 5 D E	5.70 ± 0.33 B C
G9c	68 ± 1 E	7.91 ± 0.11 E
G4	44 ± 7 F	7.30 ± 0.09 D E
G6	41 ± 6 F	7.73 ± 0.25 D E
Sterile control	37 ± 5 F	7.42 ± 0.05 D E
G12	35 ± 3 F	7.88 ± 0.36 E
G2	35 ± 8 F	7.02 ± 0.24 D

[a]Each culture was incubated in D2 medium (see Methods) for 15 days.
[b]Including sterile controls, ordered by significance groupings[d].
[c]Average of duplicate experiments.
[d]Standard deviation.
[e]Mean values followed by the same letter are not significantly different by Tukey's test ($\alpha \leq 0.05$).

Sulfate-Reducing Corrosion Experiments

Whether the cultures were grown with or without lactic acid, high-carbon steel coupons showed no loss of weight in the first three days. This may be related to deposition of salts on the metal surface, the composition of which was not determined. In the presence of lactic acid, salt deposition systematically formed a black film on the surface of the high-carbon steel coupon when subjected to SRB-M. This finding is consistent with layers of FeS that were reported by King et al. (1976). A second type of salt deposition, small uniform crystals, occurred on the coupons in the salt rich medium V9, including in the sterile controls. Three days after inoculation with SRB-M in the presence of lactic acid, the coupons showed corrosion rates with an average MIC factor of 0.28 (Figure 2), indicative of a protective effect, consistent with the results obtained by King et al. (1973, 1976) in a medium of low ferrous iron content similar to V9. In the absence of lactic acid, however, coupon corrosion rates in the SRB-M cultures were similar to those in the non-inoculated medium. After 42 days, it was possible to isolate SRB-M only from the inoculated medium supplemented with lactic acid. In contrast, cultures without lactic acid did not produce a black precipitate of ferrous sulfide, otherwise indicative of sulfate-reducing bacterial activity in culture medium V9. In our alkaline conditions, SRB-M was not able to use H_2, coming from the cathodic depolarization of the high-carbon steel, as an energy source. However, in a previous experiment, this strain was able to grow in an atmosphere of H_2 as the sole energy source. It is possible that, under alkaline conditions, SRB-M did not colonize the metal surface, alternatively, H_2 produced by cathodic depolarization may have been insufficient to support the growth of SRB-M.

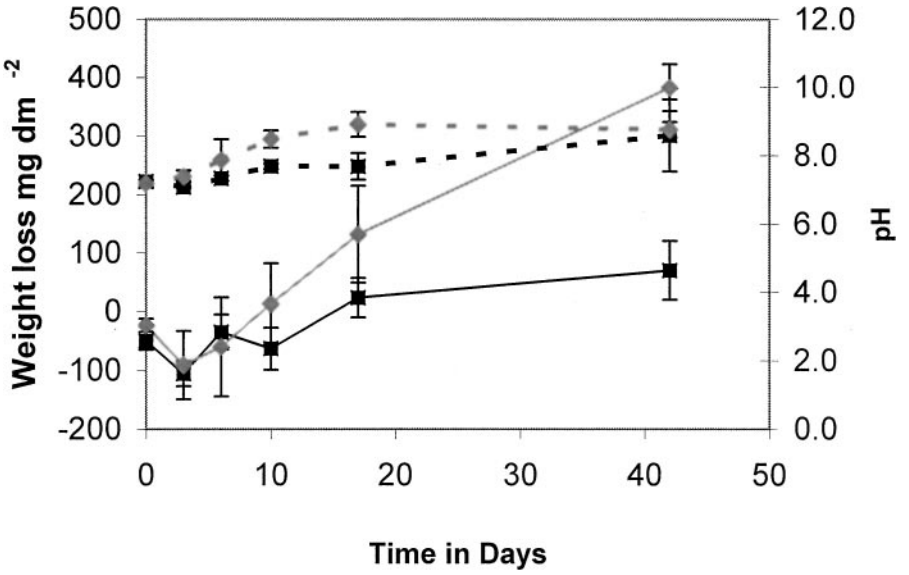

FIGURE 2 Weight loss of high-carbon steel in culture medium V9 (with lactic acid) inoculated with SRB-M ■ or uninoculated ◆ (solid lines) and pH of the culture medium (broken lines). The negative corrosion rates were produced by salt deposition on the coupon surface, the chemical nature of which was not determined.

Corrosion Experiments with Reconstructed Thermophilic Communities

Two model thermophilic bacterial communities were experimentally constructed using isolates with diverse phenotypes.

A corrosion-induction factorial experiment was carried out in culture medium D2 using high-carbon steel. The members of reconstructed community I were cultivated in all possible mixes. With the exception of G8a, each of the isolates showed a statistically significant participation in corrosion induction: G2 and G9a and G11 (all $P \geq 0.001$) showed positive corrosion influences. SRB-M, on the other hand, produced a protective effect against corrosion ($P \geq 0.001$). The mixed culture interactions G2 × G9a ($P \geq 0.001$), G9a × G11 ($P = 0.008$), and G2 × G9a × SRB-M ($P = 0.043$) were statistically significant. G2, G9a, and G11 monocultures did not influence coupon weight losses significantly beyond the effects of the sterile controls, whereas SRB-M monocultures had a protective effect (Table 2). The highest coupon weight losses occurred with the G2 × G9a interaction. Coupon weight gains (Table 2) occurred as a result of salt deposition, the chemical nature of which was not determined; its appearance indicates that it was ferrous sulfide.

Patterns of corrosion induction by communities I (without isolate G8b) and II were observed over time. The concentration of ferrous ions in the culture medium, as well as HS-evolution, pH, and corrosion rates were determined. Both communities showed similar results: the corrosion increments were approximately linear over time. The slopes (mg dm^{-2} d^{-1}) were 4.8 ($r^2 = 0.61$) and 5.0 ($r^2 = 0.64$) times greater (I and II, respectively) than the sterile control slope ($r^2 = 0.77$) which had a value of 1.24 mg dm^{-2} d^{-1} (Figure 3A). The early Fe^{2+} concentrations in the culture medium of both communities were higher than in the sterile controls. In the first 12 days of incubation, very high concentrations of Fe^{2+} were reached in the culture medium. These concentrations continued to increase only marginally in the case of community I, and by about 50% in the case of community II. This Fe^{2+} concentration increment did not show a direct relationship with corrosion of

TABLE 2 Final corrosion of coupons high-carbon steel incubated in mixed cultures of thermophilic isolates[a]

Isolates combinations[b]				Final weight loss (mg dm^{-2})
G2	G9a	G11		210[c] ± 20[d] A[e]
G2	G9a			207 ± 28 A B
G2	G9a	G11	SRB-M	174 ± 17 A B C
G2	G9a		SRB-M	95 ± 15 A B C D
	G9a	G11		92 ± 34 B C D
		G11		76 ± 12 C D
G2			SRB-M	70 ± 20 C D
G2				62 ± 36 C D
	Sterile control			48 ± 2 D E
G2		G11	SRB-M	46 ± 5 D E
	G9a			38 ± 29 D E
G2		G11		35 ± 15 D E
		G11	SRB-M	1 ± 2 D E F
	G9a	G11	SRB-M	−4 ± 3 D E F
	G9a		SRB-M	−56 ± 1 E F
			SRB-M	−81 ± 9 F

[a] In each case the isolates were incubated for 15 days.
[b] Including single-isolated and sterile controls, ordered by significance groupings[d].
[c] Average of quadruplicate experiments.
[d] Standard deviation.
[e] Mean values followed by the same letter were not significantly different by Tukey's test ($\alpha \leq 0.05$).

the coupons, showing that factors other than carbon steel dissolution affected ferrous ion concentration. The theoretical maximum relationship of Fe^{2+} formed to mg coupon weight loss (equivalent to 0.51 m*M* in 35 mL of culture medium) was almost reached at day 12 in both community cultures, and then diminished and remained at low levels (Figure 3B). In contrast, the relationship of Fe^{2+} formed to coupon weight loss increased slowly throughout the experiment in the sterile controls. These relationships allow an assessment of the Fe^{2+} content of the culture medium as an indicator not only of the breakdown of the carbon steel but also of iron reducing activity by the reconstructed consortia.

Discussion

The emphasis in MIC literature has been on SRB as corrosive agents, although a diversity of corrosion-inducting bacteria are known to exist, for example thermophilic strains. In this work, bacterial isolates from a hot spring in Guanajuato State, Mexico, belonging to the *Bacillus/Clostridium* group were used to test MIC. Results on the corrosion capability showed a tendency to be greater in consortia than in monoaxenic cultures. Similar observations were reported by Dowling et al. (1991).

It is noteworthy that isolates of the genus *Bacillus*, which is not commonly recognized as a genus containing corrosion inducers, showed a significant role in MIC in reconstructed consortia growing in microaerobic conditions. *Bacillus* strains are generally regarded as aerobic (Ford and Mitchell 1990). With our strains, MIC seemed to be related to ferric reduction, acid production and, possibly, oxygen depletion.

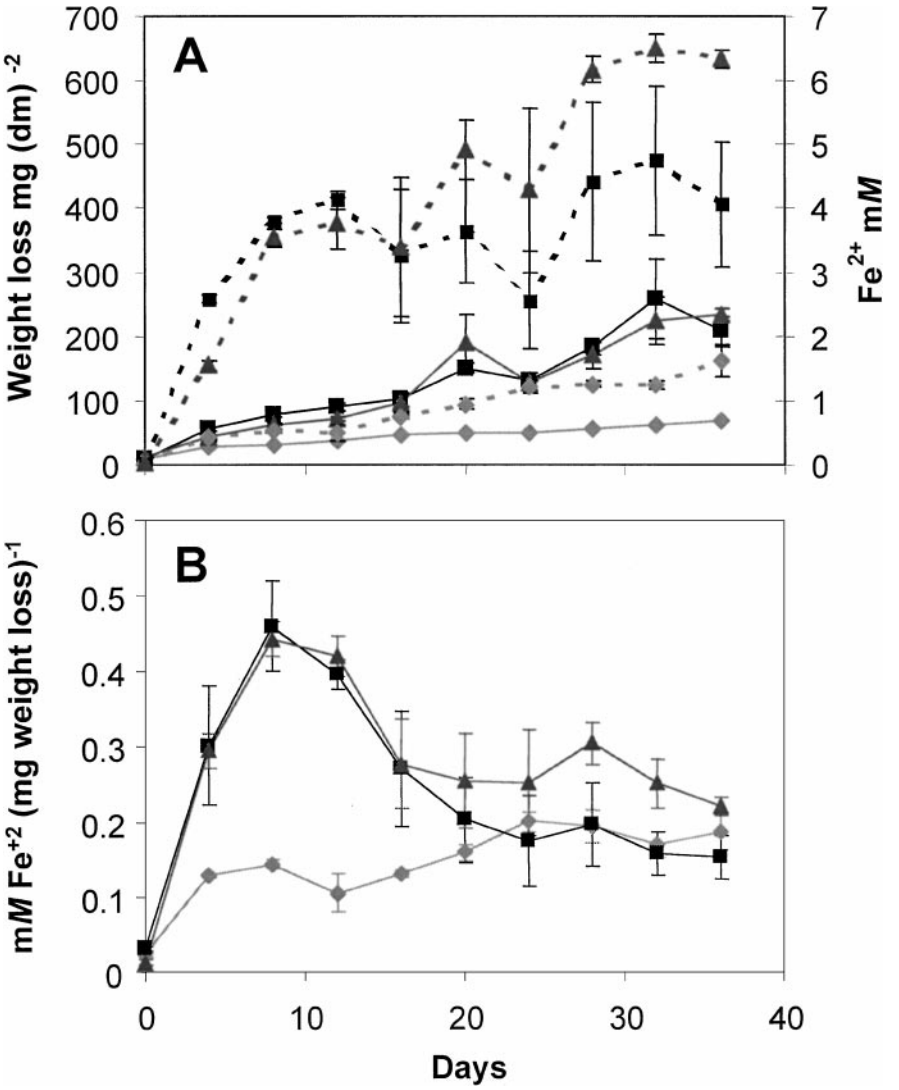

FIGURE 3 Corrosion of carbon steel coupons by thermophilic bacterial communities models I and II and its relationship with Fe^{2+} concentration in the D2 culture medium. Panel A shows coupon weight loss (solid lines) and culture medium Fe^{2+} concentration (broken lines) for model I (■), model II (▲), and sterile controls (◆). Panel B shows the change in Fe^{2+} concentration as a function of coupon weight loss in the culture medium over time. The error bars represent standard deviations.

Sulfate reducing bacteria are known to exist in bacterial communities characterized by oxic-anoxic interfaces, such as slurries and biofilms (Lovley and Phillips 1987; Okabe et al. 1999).

In previous studies, it has been pointed out that SRBs influence corrosion by consuming H_2 adsorbed to the metal surface (von Wolzogen and van der Vlugt 1934; Iverson 1966). However, an association between SRB and MIC was not consistently established in these reports. This lack of correspondence has been explained on the basis of SRB-strain differences in H_2 consumption (Bryant et al. 1991). In fact, the identification of corrosion-inducing

ability is typically by means of probes directed at various hydrogenase genes (Voordouw et al. 1990).

In this study, the sulfate-reducing ability of the SRB-M isolate was verified with hydrogen as the electron source. However, SRB-M was unable to reduce sulfate with high-carbon steel as the electron source. Reports by Daniels et al. (1987) and Boopathy and Daniels, (1991), working with methanogens in comparable conditions, showed contrasting results. However, other reports showed that SRB without hydrogenase activity can induce corrosion-rates similar to SRB with hydrogenase activity (King et al. 1973). Our data showed that detection of hydrogen-consuming capability by SRB is not necessarily indicative of corrosive ability.

With lactate as energy source, SRB-M did not enhance corrosion of high-carbon steel, instead, a protective effect (MIC factor of 0.28) was observed. Sulfide-generating activity by SRB-M in a culture medium with traces of ferric iron, produced a black precipitate. Similarly a black adherent film was also observed, as reported by King et al. (1973, 1976) for comparable conditions. King and Wakerley (1973) pointed out that ferrous sulfide prepared with an excess of sulfide ions can almost completely inhibit corrosion.

The corrosion effect of SRB-M was significantly modified by the presence of "G" isolates in the consortia. The isolates in our thermophilic bacterial community model I were not selected on the basis of their corrosion-inducing capacity, but for their range of metabolic abilities. Although various "G" isolates showed no significant high-carbon steel corrosion, their interactions in coculture were clear. In the strain-combination studies, corrosion was enhanced by the presence of strain G2 (a ferric reducing isolate). This enhancement was particularly marked in the interaction with G9a (a fermenting slime- forming isolate). It is noteworthy that SRB-M increased corrosion only when in co-culture with G2.

With strain combinations in which a FeS film was observed, the corrosion was slight. Studies of this film by King et al. (1976) showed a massive loss of the protective mackinawite sulfide film by conversion to smithite when ferrous ion was increased experimentally from 25 to 1025 μM in SRB cultures. The protective effect of the SRB-M that is consistently observed in monoaxenic cultures was eliminated by the G2 $\times$ G9a interaction (Table 2).

Similarly, Obuekwe et al. (1981a, 1981b, 1981c) and Little et al. (1998) showed that elimination of the ferric iron protective film on the steel surface by ferric reducing bacteria resulted in exposure of a new surface and, consequently, in added corrosion. We observed that the G2 $\times$ G9a interaction, in the absence of SRB-M, also led to an increase in corrosion, in comparison with the control (Table 2), which also may be explained in terms of the MIC mechanism by ferric-reducing bacteria.

Ferrous ion concentrations in reconstructed consortia reached values of 1 mM almost immediately and more than 6 mM after 12 days of incubation (Figure 3). The sulfide concentrations determined in both bacterial communities were usually less than 40 μM and never higher than 90 μM. In those ferrous ion and sulfide concentrations, SH^- produced by SRB-M is expected to react with dissolved Fe^{2+} forming a flocking nonadhesive product (King et al. 1973). Therefore, the protective sulfide film is not formed or is removed, and new metal surface is constantly re-exposed, resulting in corrosion rates of 4.8- to 5-fold higher than those in the sterile controls.

The Fe^{2+} concentration/steel-weight-loss ratio was employed as an indicator of ferric reducing activity in both bacterial communities. This comparison implies that the ferrous ion accumulation in the culture medium of both bacterial communities was due not only to steel breakdown but also to a rapid bacterial ferric reduction. The maximum theoretical ratio of Fe^{2+} and carbon steel weight loss is 0.51 mM mg-1 in our culture volume of 35 mL. However, the actual amount may be affected by ferrous ion reoxidation, ferrous reaction with sulfide ions, ferrous attachment to bacteria or the carbon steel surface, and/or by precipitation

due to solubility limits. It is expected that reduction of ferric iron from deoxidized ferrous iron will maintain high ferrous concentrations. The ratios ferrous concentration/weight loss in both bacterial communities differed from those of the sterile controls, especially in the first days of the experiment when the maximum theoretical value was reached (Figure 3B). Later, the Fe^{2+} concentrations remained relatively constant, probably because of solubility limits in the organic-rich medium. It is important to note that as corrosion progressed, the Fe^{2+}/steel weight loss rates values diminished to approximately those of the controls. In fact, the ferric-reducing consortia maintained ferrous concentrations at high levels.

It was interesting to observe that, in monoaxenic culture, isolate G2 did not show MIC factors significantly different from those of the sterile controls. However, when growing together with G9a, the latter seemed to cause bacteria to adsorb on the metal surface and deplete oxygen until anoxic conditions prevailed. In the thermophilic bacterial community "model II," the fermenting G9a and the aerobic G11 isolates from model I, were replaced with isolates G8b (anaerobe growth only in the presence of nitrate) and G9c (fermenting, slime former). The substitution of these isolates, resulted in similar effects on MIC and a comparable effect on ferrous ion accumulation over time to the thermophilic bacterial community "model I." The fermentative isolates G9a and G9c (both taxonomically related to *Bacillus licheniformis*), both of which have an agglutinating function, seemed to work similarly in both reconstructed consortia. It is interesting that the inclusion of G8b had no effect on corrosion. Similar results were obtained by inclusion of G8a (nitrate response) in consortia I.

Our data show that bacterial strains with hydrogenase activity were not necessarily corrosive and that corrosion induction was modified substantially by the metabolic background of the whole bacterial community, particularly by the presence of ferric-reducing members. Furthermore, ferrous sulfide film formation appeared to be affected not only by SRB activity, but also by ferric-reducing activity. The latter seems to enhance the corrosion rate.

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