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Accelerated cathodic reaction in microbial corrosion of iron due to direct electron uptake by sulfate-reducing bacteria

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ABSTRACT

Microbially influenced iron corrosion by sulfate-reducing bacteria (SRB) is conventionally attributed to the chemical corrosiveness of H_2S, facilitated abiotic H^+-reduction at deposited FeS, and biological consumption of chemically formed ('cathodic') H_2. However, recent studies with corrosive SRB indicated direct consumption of iron-derived electrons rather than of H_2 as a crucial mechanism. Here, we conducted potentiodynamic measurements with iron electrodes colonized by corrosive SRB. They significantly stimulated the cathodic reaction, while non-corrosive yet H_2-consuming control SRB had no effect. Inactivation of the colonizing bacteria significantly reduced current stimulation, thus confirming biological catalysis rather than an abiotic cathodic effect of FeS.

1. Introduction

Whereas iron corrosion by oxygen from air is, to our present knowledge, a purely electrochemical process, iron corrosion in neutral media in the absence of air (as, for instance, in aqueous underground or inside iron pipes) is largely biologically influenced. Sulfate-reducing bacteria (SRB) are commonly considered to be the main originators of this microbiologically influenced corrosion (MIC) [1,2]. SRB gain their biochemical energy for growth by reducing sulfate (SO_4^{2-}) to sulfide (H_2S, HS^-) with natural organic compounds as electron donors that are oxidized to CO_2 (also referred to as sulfate respiration). In addition, many SRB can also utilize molecular hydrogen (H_2), a common product of other bacteria involved in the biological breakdown of organic compounds in oxygen-free aquatic systems such as sewers, sediments and swamps.

The mechanisms by which SRB act upon metallic iron have been controversially discussed in literature [3–6]. The basic feature of previously described models is always the low-potential electron release by the metal according to

$$Fe^{2+} + 2e^- \rightleftarrows Fe$$

$$E_{SHE,298K} = -0.47 + 0.0296\ \log(a_{Fe^{2+}}) \quad (1)$$

(the previous redox potential ($E^0_{SHE} = -0.44$ V) has been revised according to [7]). The sulfide formed by SRB behaves as a chemically aggressive compound [3,8,9], resulting in the bulk equation $Fe + H_2S \rightarrow FeS + H_2$; for review see also Ref. [2]. In this way, SRB act indirectly by chemical reaction of their metabolic end product (chemical microbially influenced corrosion, CMIC).

A fundamentally different, traditional mechanistic proposal is based on the inherent ability of many SRB to utilize H_2 as electron donor ($4H_2 + SO_4^{2-} + 2\ H^+ \rightarrow H_2S + 4H_2O$). Reduction of protons in water to hydrogen according to

$$2H^+ + 2e^- \rightleftarrows H_2$$

$$E_{SHE,298K} = 0.00 - 0.0296\ \log(a_{H_2}) - 0.0592\ pH \quad (2)$$

can in principle be linked with iron oxidation (Eq. (1)) and results in the net reaction

$$Fe + 2H^+ \rightarrow Fe^{2+} + H_2. \quad (3)$$

Early investigators speculated that in the absence of microorganisms, the H_2 formed builds up a 'hydrogen film' at the metal surface, ultimately impeding reaction (3) and thus iron dissolution to progress [4,10]; traditionally, this impediment is often referred to as 'polarization'. In the presence of microorganisms with the capability of H_2 utilization such as SRB, their effective scavenging of H_2 was suggested to lower the local partial pressure and through such 'depolarization' allow iron dissolution to proceed. This proposal became therefore known as 'cathodic depolarization theory'.

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Because H_2S combines with Fe^{2+} ions from the primary dissolution (Eq. (1)), the net reaction (including bicarbonate that is readily available in many water systems) is

$$4Fe + SO_4^{2-} + 3HCO_3^- + 5H^+ \rightarrow FeS + 3FeCO_3 + 4H_2O. \quad (4)$$

From a merely thermodynamic perspective, however, the above considerations may be questioned. The redox potential of the electron donor (Eq. (1)) is more negative than that of the electron acceptor (Eq. (2)). Accordingly, the free energy of reaction (3) under standard conditions (except that $(a_{H^+} = 10^{-7})$ is $\Delta G^0_{pH7} = -10.6$ kJ mol^{-1}, and the reaction can, in principle, proceed spontaneously. At environmentally relevant activities of Fe^{2+}(aq) that are significantly below standard activity, the Fe^{2+}/Fe redox couple is even more negative, often $E_{environ} \leqslant -0.6$ V vs. standard hydrogen electrode (SHE), so that $\Delta G_{environ} \leqslant -36.7$ kJ mol^{-1}. For a thermodynamic halt ($\Delta G \geqslant 0$) of iron dissolution according to reaction (3), one would have to assume a 'hydrogen film' with a local fugacity corresponding to $p_{H_2} > 10^{11.3}$ Pa. Considering the extremely fast diffusion of H_2, viz. of the hydrogen species that is used by bacteria, such local build-up of a hydrogen film appears very unrealistic. If SRB have a direct influence on corrosion, an understanding can be only expected from the viewpoint of electrokinetics, in particular of H^+ reduction to H_2, rather than *via* mere thermodynamic considerations. H_2 formation on iron in circumneutral water is inherently slow, a 'kinetic bottle neck' due to limitations in proton availability and combination reactions forming H_2 [11–13]. Still, several hydrogenase-positive cultures of sulfate-reducing bacteria apparently stimulated the cathodic current ('depolarization') on mild steel electrodes [5,14,15]. The authors attributed this to bacterial H_2-uptake from the electrode surface and, hence, interpreted the observation in favor of the 'classical' depolarization theory. The ability of SRB for scavenging H_2 from corroding iron and water has indeed been shown [6,16,17].

However, an experimental misconception in the early electrochemical study of the postulated direct mechanism involving H_2 with conventional SRB strains was the addition of lactate, a routine, excellent cultivation substrate of SRB [5,14,15]. Lactate represents a competitive electron donor in addition to 'cathodic' H_2 and, more importantly, leads to excessive concentrations of aggressive sulfide causing chemical corrosion (CMIC) and altering the electrode surface drastically. Costello [3] and Hardy [6] therefore omitted lactate and gave proof that cathodic depolarization did not occur in SRB cultures with metallic iron as the only source of electrons for the organisms; rather, acceleration of the cathodic reaction was shown to result from the reactivity of dissolved sulfide. Accelerated corrosion due to bacterial H_2-uptake from the metallic iron surface was consequently questioned by several authors, particularly as SRB incubated with iron alone did not accelerate corrosion [18–20].

In another model of SRB-induced corrosion, stimulation of H^+-reduction to H_2 by catalytically active ferrous sulfides on the iron electrode was suggested [21,22]. Hence, SRB were thought to scavenge H_2 from FeS rather than from the metallic surface. Chemically prepared, fine suspensions of FeS transiently accelerated the cathodic reaction and iron loss even in the absence of bacteria, viz. if H_2 was not consumed [22,23]. However, a variety of both amorphous and crystalline iron sulfides exist which exhibit very different properties with regard to the corrosion of iron. Neither their properties nor the extent of their contribution to anaerobic iron corrosion are completely understood at the moment [24].

In another approach towards a mechanistic understanding of anaerobic corrosion, SRB were directly enriched and isolated with metallic iron as the only source of electrons (viz. without an organic substrate such as lactate) for sulfate reduction [19]. They severely corroded the metallic substrate with a rate of up to 0.7 mm Fe^0 yr^{-1}, corresponding to 61 μA cm^{-2} [25]. The corrosion rate could not be explained by dependency on H_2, the chemical formation of which from iron and water was by far too slow [19,25]. This and the significant conductivity of the deposited FeS-containing crust [25] indicated a direct electron uptake (i.e., electrical microbially influenced corrosion, EMIC) by the attached cells according to

$$8e^- + SO_4^{2-} + 10H^+ \rightarrow H_2S + 4H_2O \quad (5)$$

$$E_{SHE,average,298K} = 0.30 + 0.0074\ \log(a_{SO_4^{2-}}/a_{H_2S}) - 0.074\ pH$$

(underlying data in Supplementary material) and thus an effective by-pass of the H_2-formation reaction. The net reaction (combination of Eq. (1) and (5)), which is the same as Eq. (4), results in significant mineral precipitation on the iron.

Such a postulated direct electron uptake urges upon corroboration by electrochemical measurements. If the novel SRB accelerate corrosion by direct electron uptake, this should be obvious from a shift of the free corrosion potential and from an increase of the cathodic current of iron electrodes in potential-controlled experiments. In the present study these effects were investigated using iron coupons colonized and encrusted (Eq. (4)) by the corrosive SRB *Desulfopila corrodens* (tentative name) strain IS4 for electrochemical measurements in defined electrolyte. No organic electron donor was added, viz. all electrons for sulfate reduction were provided through the metal. Moreover, to distinguish between the impact of bacterial activity and their deposited iron sulfides, the current–potential relationship was measured prior to and after chemical inactivation of the colonizing bacteria. *Desulfovibrio* sp. strain HS3, an organism similar to SRB investigated in former 'depolarization' studies and growing well with H_2 served as control culture. The expected electrokinetic effects of strain IS4 were indeed observed, thus fully supporting the postulated enhancement of corrosion by direct biological electron uptake rather than by H_2-consumption.

2. Materials and methods

2.1. Chemicals and organisms

All solutions and culture media were prepared from chemicals of analytical grade and ultrapure deionised water (Purelab Plus by Elga Labwater, Celle, Germany). Culture liquids were sterilized in an autoclave at 121 °C for 25 min. Pre-cultures of the two isolated SRB strains used in this study, *D. corrodens* strain IS4 and *Desulfovibrio* sp. strain HS3 [19,25], were incubated in butyl-rubber-stoppered glass bottles with artificial seawater medium (ASW) [26] buffered by CO_2/$NaHCO_3$, and provided with an anoxic headspace of CO_2/N_2 (10:90, v/v). ASW contained typically 28 mM sulfate as an electron acceptor and no oxidizable organic substrates. For the experiments including sulfate analysis, the sulfate concentration was lowered to 5 mM for more precise detection of its consumption. Pre-cultures of SRB strains were grown on H_2 and subsequently flushed with CO_2/N_2 for 30 min to prevent transfer of dissolved sulfide and H_2 into the incubations. The cell density in inocula was determined by acridine orange (0.1 mg ml^{-1}) staining and epifluorescent microscopy (Zeiss Axiophot, Carl Zeiss MicroImaging, Göttingen, Germany), so that all experiments could be started with identical cell numbers.

2.2. Free corrosion potential and potentiodynamic measurements

2.2.1. Electrochemical cell setup and incubation

Electrochemical cells were constructed as follows: Sheets of pure iron (composition in wt.%: 99.877% Fe; <0.06% Mn, <0.03%

Cu, <0.01% C, <0.005% P, <0.005% N, <0.005% Co, <0.005% Sn, <0.003% S) were mechanically ground, cut into coupons of the dimensions depicted in Fig. 1, and contacted with a silver wire (0.5 mm in diameter). Coupons were then cleaned with alkali soap and pure ethanol, sterilized by complete immersion in pure anoxic ethanol for 90 min and subsequently dried in a stream of N_2.

Duran glass bottles (250 mL) filled with 200 mL of anoxic sterile ASW (electrolyte) were used as electrochemical cells (Fig. 1). Sodium sulfide (0.1 mM) was added as reducing agent to remove traces of residual oxygen and shorten the *lag*-phase of cultures. Each bottle was then equipped with a magnetic stir bar and the two sterilized iron coupons (WE and CE) in such a way that only the lower 5 cm of the coupons were immersed. Silver wires providing electrical contact to the coupons were pierced through the butyl-rubber septum. For connection with the reference electrode, a sterilized steel cannula (13.201 stainless steel; Unimed S.A., Lausanne, Switzerland) of 0.8 mm in diameter was introduced into the cell through the top septum and connected to a glass holder *via* gas-tight butyl rubber tubing (not shown). The glass holder contained two compartments separated by a glass diaphragm. The electrode compartment of the glass holder was filled with a 3 M KCl electrolyte and equipped with a standard Ag/AgCl reference electrode (type 6.0750.100 by Metrohm AG, Herisau, Switzerland). To maintain ionic conductivity between the main cell and the reference electrode compartment, ASW was sucked into the compartment so as to establish the ionic connection and held in place by a stopcock. The low volume to electrode surface ratio of around 5 mL cm^{-2} allowed reliable determination of sulfate consumption and ferrous iron formation.

Three electrochemical cells were prepared for each strain, i.e. six in total, and inoculated to initial cell densities of 10^6 cells mL^{-1}. Sodium acetate (1 mM) was added to cultures of heterotrophic strain HS3. Electrochemical cells were gently stirred (250 rpm) during incubation at 28 °C. One culture of each strain was used for the potentiodynamic measurements directly after inoculation and a second culture after five days when the coupons with strain IS4 were just completely covered with black precipitate. The third culture of each strain was first used to record the development of the free corrosion potential over eight days and subsequently used for potentiodynamic measurements (see also Fig. S1). All potential-controlled measurements included chemical sterilization and thus sacrificed the colonized electrodes.

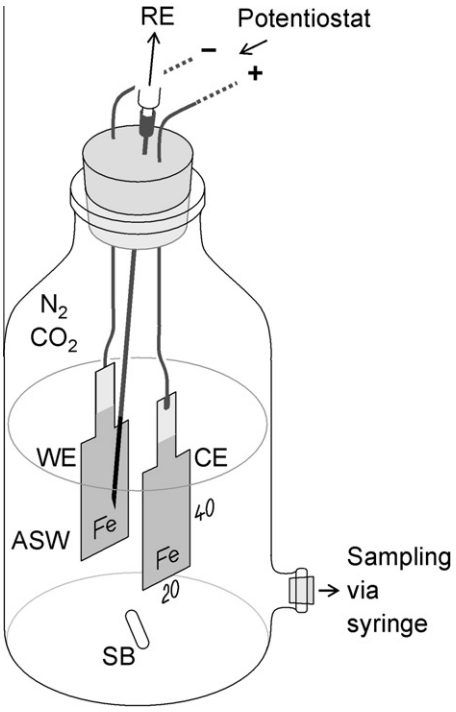

Fig. 1. Electrochemical cell for potential-controlled experiments. WE, working electrode (iron); CE, counter electrode (iron); RE, sterilized steel cannula as contact to the external reference electrode; ASW, artificial sea water medium; SB, magnetic stir bar. Anoxic headspace consists of CO_2/N_2 (10:90, v/v). Dimensions of electrodes (in mm) are identical for WE and CE.

2.2.2. Determination of free corrosion potential

The free corrosion potential, E_{corr}, was followed by connecting the electrodes of the electrochemical cells during their incubation with strains IS4 and HS3 *via* a multiplexer (ECM 8 by Gamry Instruments) to a Reference 600 potentiostat (Gamry Instruments, Warminster, PA, USA).

2.2.3. Potentiodynamic measurements – Linear sweep voltammetry

Before measurement, the precipitate-covered electrodes were transferred into fresh electrolyte to avoid an impact of the changes in the electrolyte due to growth so as to ensure reproducible starting conditions. The electrolyte for the measurements was sterile basal ASW without vitamins and trace elements. All transfers were conducted in the N_2 atmosphere of a glove box to prevent oxidation processes at the encrusted electrodes. Electrodes of the electrochemical cell were then connected to a CompactStat potentiostat (Ivium Technologies B.V., Eindhoven, The Netherlands). Once E_{corr} had become constant, a cathodic potential sweep was performed over a range of $\Delta E = -400$ mV at a rate of 1 mV s^{-1}, starting at E_{corr}.

Immediately afterwards, electrodes were disconnected and sterilized with 0.3% (v/v) glutaraldehyde. This concentration had been proven in separate experiments (Fig. S2) to be sufficient for complete inhibition of the activity of strain IS4. To ensure inactivation also of cells inside the possibly protective massive corrosion crust that had been formed after eight days in culture of strain IS4, the glutaraldehyde concentration was doubled for these electrodes. Electrodes were sterilized in the glutaraldehyde-containing medium at 6 °C for 12 h, washed twice thoroughly in anoxic basal ASW, and again transferred into fresh anoxic ASW electrolyte. Washing steps and transfers were again performed under N_2 atmosphere in a glove box. Subsequent cathodic potential sweep of the sterilized electrodes was performed and recorded as before.

Control measurements were conducted in two electrochemical cells without inocula. One cell was filled with sterile anoxic ASW as sterile control, while the other cell was filled with anoxic ASW containing 1 mM sodium sulfide. The electrochemical control cells were incubated for four days until dissolved sulfide had completely reacted with the iron electrode and could not be detected any more in the sulfide-amended control. Potentiodynamic measurements were carried out as described above. Another separate electrochemical cell was incubated under aseptic conditions to monitor abiotic hydrogen formation.

2.3. Chemical analyses

A butyl-rubber-stoppered sampling port allowed for anaerobic and aseptic withdrawal of culture medium during incubation of electrochemical cells. Samples were immediately filtered (Acrodisc 13 mm syringe filter with 0.45 μm Nylon membrane by Pall Life Sciences, Port Washington, NY, USA) to remove FeS particles and then analyzed for concentrations of sulfate and dissolved ferrous iron. The pH was measured in a separate, fresh sample with a SenTix MIC-D pH-sensitive electrode (WTW GmbH, Weilheim, Germany). Ferrous iron was quantified by inductively coupled plasma optical emission spectroscopy (ICP-OES; IRIS Intrepid HR Duo by Thermo Fisher Scientific, Waltham, MA, USA). Sulfate was quantified by ion chromatography (Metrohm 761 Compact IC,

Metrohm AG, Herisau, Switzerland) with a conductivity detector. Ions were separated *via* a Metrosep A Supp 5–100 column with an eluent of 3.2 mM Na_2CO_3 and 1 mM $NaHCO_3$ at a flow rate of 0.7 mL min^{-1}. Hydrogen was quantified from headspace by gas chromatography with thermal conductivity detection (Shimadzu GC 8A, Shimadzu, Kyoto, Japan) and a Porapak Q N80/100 column (Machery-Nagel, Düren, Germany; 40 °C, N_2 as carrier gas). Expected sulfide production from bacterial scavenge of H_2 in electrochemical cells was calculated assuming the stoichiometry

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\,H_2O.$$

2.4. Electrochemical impedance spectroscopy

To reveal the dielectric vs. conductive properties of the corrosion crust, electrochemical impedance spectroscopy (EIS) was performed with iron electrodes in the form of wires that were incubated with strains IS4 and HS3, as well as in sterile ASW as the electrolyte. The setup of the electrochemical cell was similar as for the potential sweep experiments, except that platinum served as counter electrode. EIS measurements were carried out using a sinusoidal signal from 10^4 to 10^{-3} Hz with an amplitude of 10 mV around E_{corr}. Impedance spectra were fitted to an equivalent circuit using the ZView EIS analysis software (Scribner Associates Inc., Southern Pines, NC, USA).

2.5. Scanning electron microscopy

Iron coupons were incubated for 6 weeks in a batch culture with strain IS4 and HS3, respectively. Specimens were prepared for scanning electron microscopy as described elsewhere [25]. SEM was performed in a Zeiss Leo 1550 FE-SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany) at extra high tension (EHT) between 5 and 15 kV.

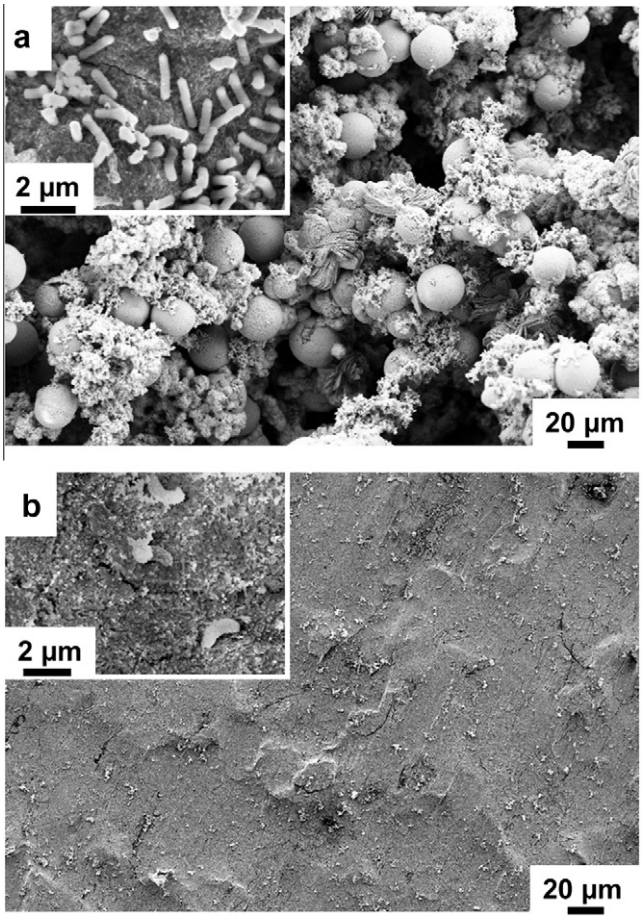

Fig. 2. Scanning electron micrographs of iron specimen surfaces after incubation for six weeks with a culture of (a) corrosive strain IS4 and (b) control strain HS3.

3. Results and discussion

Evidence for microbially enhanced corrosion of iron due to direct electron uptake (Eq. (3)) and by-pass of the abiotic hydrogen formation was hitherto based on the much faster growth of novel sulfate-reducing bacteria with iron in comparison to conventional H_2-consuming strains [19,25], and the significant electrical conductivity of the mineral crust covering the corroding metal [25]. If the slow abiotic reduction of water-derived protons (Eq. (2)) is indeed largely overlaid by a faster biological cathodic reaction (Eq. (3)), this should be also obvious from electrochemical measurements with iron electrodes in cultures of corrosive strains and comparison with sterile controls and non-corrosive, conventional strains. First the influence of *D. corrodens* strain IS4 on the free (mixed) corrosion potential (E_{corr}) was measured. Subsequently, the cathodic reaction (Eq. (3) vs. (2)) was characterized

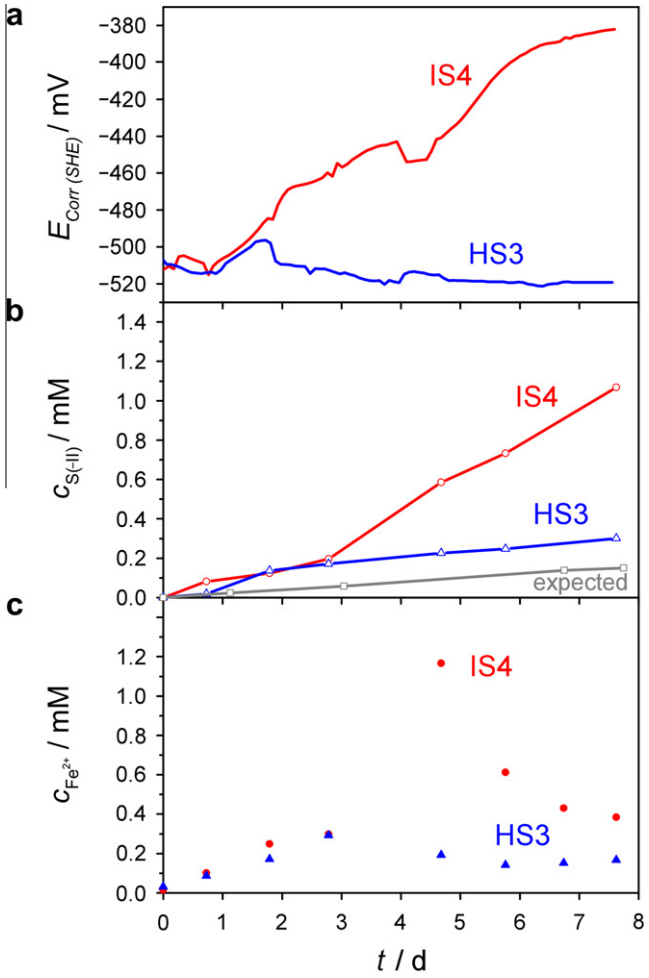

Fig. 3. Study of the corrosiveness of strain IS4 (red) and strain HS3 (blue; control strain) by incubation in electrochemical cells. (a) Continuous measurement of E_{corr}; values vs. standard hydrogen electrode (SHE) (b) Reduction of sulfate displayed as production of sulfide as an indicator of bacterial activity; expected values (grey) display calculated sulfide production solely from usage of chemically formed hydrogen. (c) Concentration of soluble ferrous iron. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

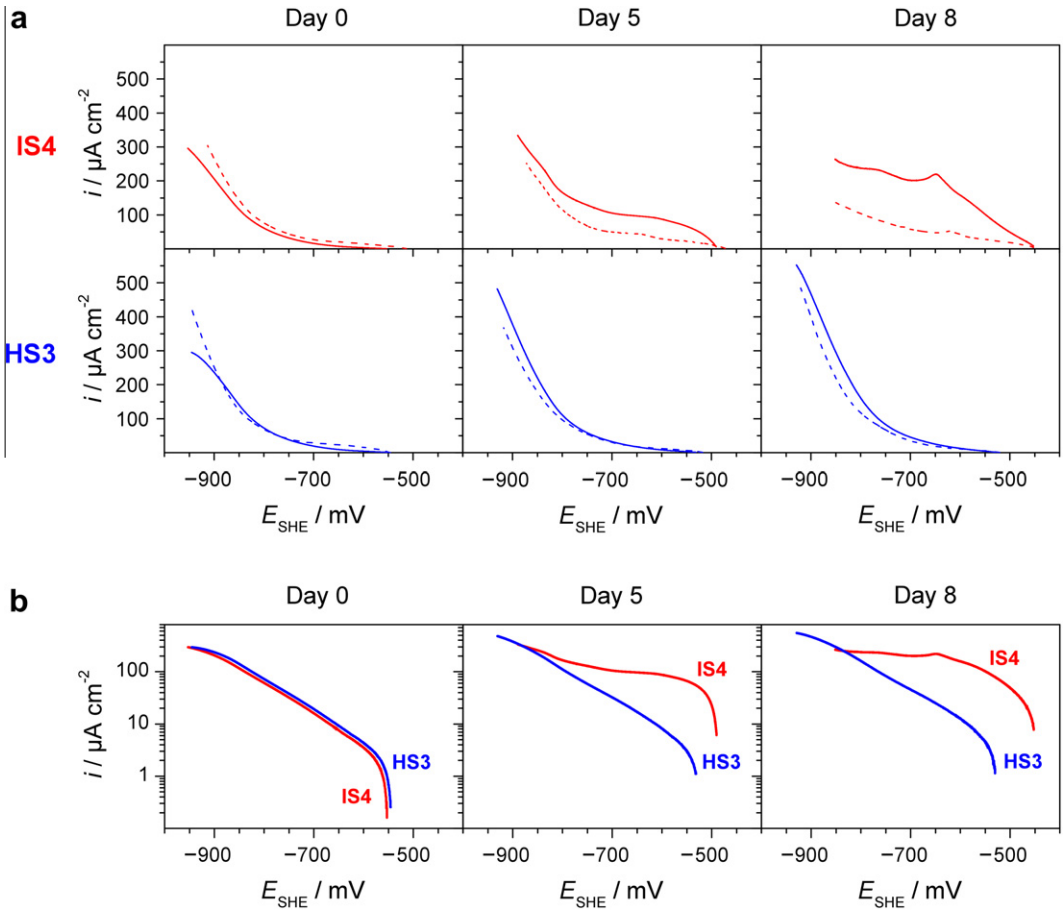

Fig. 4. (a) Cathodic current density (i) vs. applied potential (displayed vs. standard hydrogen electrode, E_{SHE}) of iron electrodes incubated briefly (day 0) and for five and eight days with corrosive strain IS4 (red) and non-corrosive strain HS3 (blue). Measurements were performed in fresh artificial seawater medium as electrolyte with viable cells (solid line) and after sterilization with glutaraldehyde (dashed line). The potential sweep with 1 mV s^{-1} ranged from the free corrosion potential (E_{corr}; start) to −400 mV below E_{corr}. (b) Comparison of voltammogramms for viable cells of HS3 and IS4 after different incubation times in a semi-logarithmic plot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

by recording the external current density in response to imposed potential shifts towards values more negative than E_{corr}. In all these experiments, the electrons for sulfate reduction were solely provided *via* iron (lithotrophic growth conditions), viz. there was no organic electron donor such as the otherwise frequently employed lactate. Finally, it was investigated whether the assumption of conductive FeS structures within non-conductive (dielectric) carbonates [Eq. (4); [25]] is corroborated by impedance measurements.

3.1. Influence of corrosive SRB on the free corrosion potential of iron

Fig. 2 visualizes the significant differences in the coverage of the iron surface in cultures of the corrosive and non-corrosive strain. Strain IS4 led to massive deposition of corrosion products while control strain HS3 caused only slight changes of the surface (Fig. 2). Cells of strain IS4 occurred embedded in and attached to corrosion products. The spherical structures (Fig. 2a) represent sulfur-free carbonates surrounded by a mixture of sulfur-containing minerals, as revealed by energy dispersive X-ray spectroscopy (not shown).

E_{corr} was continuously monitored during incubation of the iron electrodes with strains IS4 and HS3 during eight days (Fig 3a). Simultaneously, the microbial activity was recorded as consumption of the electron acceptor (Fig. 3b), sulfate, that is stoichiometrically converted to and precipitated as ferrous sulfide [19,25]. The observed sulfate consumption was compared to a calculated

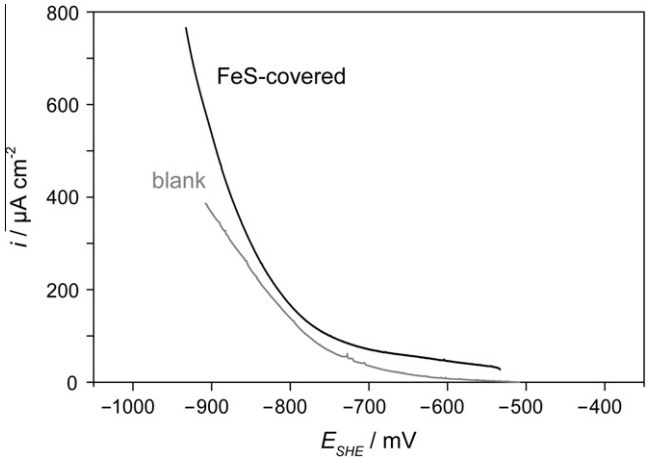

Fig. 5. Voltammogramms recorded for a sterile blank and FeS-covered iron electrode in fresh sterile anoxic ASW.

(theoretical) sulfate consumption that would occur solely by utilization of the abiotic 'cathodic' H_2 (0.25 mol SO_4^{2-} per mol H_2), the formation of which was measured with iron in sterile medium. The free corrosion potentials in the two cultures began to diverge considerably after two days of incubation. The increasing shift of E_{corr} by strain IS4 towards less negative values is in full agreement

with an increasing dominance of the biological (cathodic) electron uptake reaction (Eq. (5)) over the abiotic proton reduction (Eq. (2)). The electron-withdrawing cells of strain IS4 obviously became more and more established and grew during the incubation period. Such a pronounced, steady shift of E_{corr} was not observed with control strain HS3. Only during the second day, strain HS3 caused a transient though rapid increase of E_{corr}. This is attributed to the formation of iron sulfide forms with temporary catalytic cathodic activity, i.e. with increased rate of abiotic H_2 formation (details in Fig. S3). This assumption is supported by the initially increased sulfate reduction that must be due to H_2 utilization. At later times, E_{corr} in the presence of the non-corrosive strain HS3 became slightly more negative and the rate of sulfate reduction was not higher than the theoretical rate expected from the simple utilization of 'cathodic' hydrogen.

Furthermore, also soluble ferrous iron was monitored during the incubation (Fig. 3c) because this is occasionally regarded as another parameter reflecting anaerobic iron corrosion. However, the initial increase of the soluble ferrous iron concentration in the incubation with strain IS4 was rather early (day 4) followed by a rapid decrease. Even though the strong initial iron(II) increase by strain IS4 (in comparison to strain HS3) does reflect the high corrosive activity, the effect is subsequently masked by precipitation. Because FeS as the most insoluble iron(II) mineral is assumed to precipitate immediately, the decrease of the iron(II) concentration must be due to subsequent precipitation of $FeCO_3$ with increasing pH. The overall corrosion process according to reaction (4) is proton consuming and leads to an increase of pH even in a buffered solution particularly near the surface of the electrode [27,28], so that the bulk pH in the present incubations of strain IS4 increased from 7.1 to 7.8. Results indicate that iron(II) monitoring is an ambiguous method for studying MIC, not only because of sulfide but also because of carbonate precipitation.

In conclusion, the pronounced differences in the development of E_{corr} in the cultures of strains IS4 and HS3 were as expected. The positive shift of the mixed potential, E_{corr} in cultures of strain IS4 reflects the postulated direct electrical coupling of the primary anodic reaction (Eq. (1)) with biological sulfate reduction (Eq. (5)) as the dominant cathodic reaction outcompeting coupling to H_2 evolution. It is true that such shifts of E_{corr} are also observed in electrochemical measurements with 'classical' SRB [29–32]; however, they are routinely provided with an organic electron donor leading to excessive sulfide. The abiotic shift of E_{corr} by sulfide is attributed to two factors, an accelerated cathodic reaction on the one hand and passivation by a particular type of the formed FeS layer on the other hand. As the strong shift in E_{corr} alone is no proof for the acceleration of the cathodic reaction [33] by direct electron uptake, this assumption was further verified by investigating this half-reaction by potentiodynamic measurements.

3.2. Revealing the microbial cathodic activity by linear sweep voltammetry

If strain IS4 significantly increases E_{corr} towards more positive potentials by catalyzing a fast cathodic reaction, this should become obvious also in the increased cathodic current upon imposing a more negative electrode potential than E_{corr} (ΔE). Such recorded current should be higher than in sterile control experiments or with non-corrosive SRB that lack the ability to directly derive electrons from iron. Furthermore, if increase of the cathodic current is due to biological rather than to abiotic catalysis, e.g. by precipitated ferrous sulfide (Eq. (4)), inactivation of the cells should decrease the cathodic current.

The first task was to search for an appropriate sterilizing procedure for the crucial control measurements with inactivated cells. Such a procedure should effectively and specifically inactivate all crust-

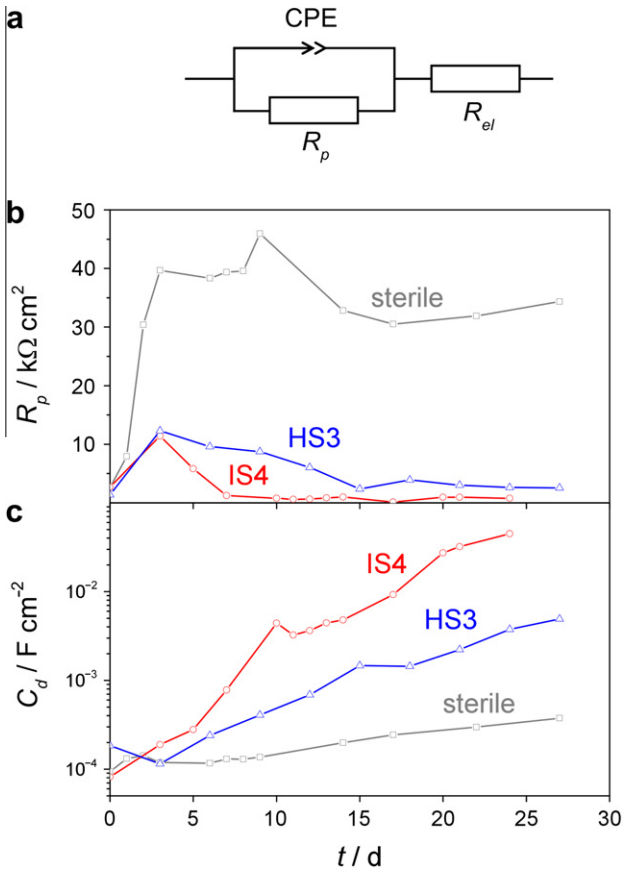

Fig. 6. Revealing the electronic properties of the sulfidic layers formed on iron wires in cultures of strains IS4 (corrosive; red) and strain HS3 (non-corrosive; blue). For comparison, measurements were also carried out with wires incubated in sterile seawater medium (grey). (a) Equivalent circuit used for calculation of polarization resistance, R_p, and capacitance, C_d; CPE, constant phase element. (b) Change of the polarization resistance during incubation. (c) Change of the capacitance during incubation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

associated bacterial cells, but should not change the electrokinetic properties of the iron surface or the inorganic crust. Heat treatment or the biocide sodium azide affected the electrokinetic behavior even of blank and sterile electrodes. Detergents (e.g. Tween-20), on the other hand, did not sufficiently inactivate cells. The best-suited sterilizing agent was glutaraldehyde. It inhibited sulfate reduction (Fig. S2) and had no significant effect on iron electrodes.

The electrodes incubated with the bacteria were transferred to fresh medium for the investigation by linear sweep voltammetry (LSV). In this way, all measurements were conducted in electrolyte of identical composition, which excludes any influence by compositional changes of the electrolyte during incubation. The LSV measurements were performed shortly after inoculation (day 0) and after five and eight days of incubation with strains IS4 or HS3 (Fig. 4). The selected scan rate of 1 mV s^{-1} is assumed to be slow enough for attached microorganisms to reach their metabolic steady state [34,35].

Briefly after inoculation, there was no significant difference in the current response to the applied potential between electrodes before and after treatment with glutaraldehyde. Neither was there a difference between the incubations with strains IS4 and HS3 (Fig. 4b). The short incubation period was obviously insufficient for bacterial attachment to the electrodes. The minor differences before and after the chemical sterilization (Fig. 4a) can be interpreted as a slight effect of glutaraldehyde. After five days of incubation with strain IS4, when the electrode had just been

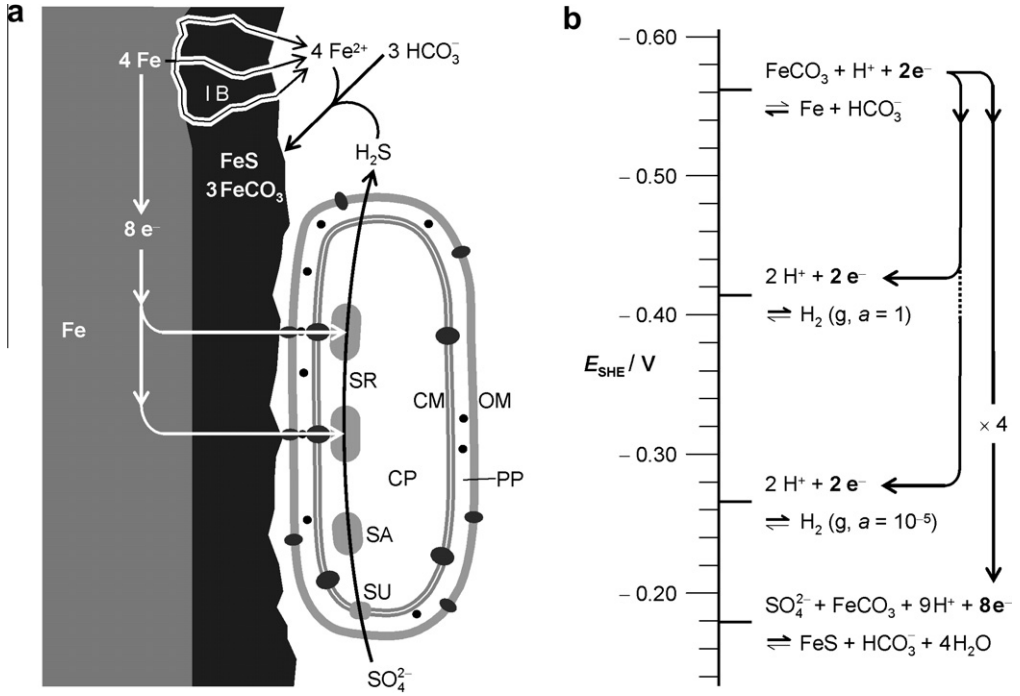

Fig. 7. Basic topological and electrochemical aspects in the study of corrosive sulfate-reducing bacteria. (a) Simplified scheme of flow of electrons from dissolving iron via conductive precipitate (FeS contained in $FeCO_3$) into the bacterial cell. Specific proteins in the outer membrane (OM), periplasm (PP) and cytoplasmic membrane (CM) allow electron transport to the enzymes for sulfate reduction (SR). The scheme also indicates ion bridges (IB), the cytoplasm (CP), enzyme for sulfate activation (SA), and protein for sulfate uptake (SU). The scheme does not include biochemical energy conservation (energetic coupling) and biosynthesis of cell mass such as CO_2 assimilation. (b) Equilibrium redox potentials of coupled half cell-reactions under the presently studied conditions of a seawater system. Equilibrium redox potentials are determined by dissolved sulfate and bicarbonate, and by crystalline ferrous carbonate and ferrous sulfide (here assumed instead of amorphous ferrous sulfide). For convenience, all redox potentials are shown for pH 7. The redox potential of $2H^+/H_2$ was calculated according to Eq. (2) for standard fugacity of H_2 (g, $a_{H_2} = 1$), and for fugacity $a_{H_2} = 10^{-5}$. The redox potential (vs. SHE/V) of the system $FeCO_3/Fe + HCO_3^-$ was calculated as $E_{298K} = -0.414 - 0.0296\log(a_{HCO_3^-}) - 0.0296$ pH. The redox potential of the system $SO_4^{2-} + FeCO_3/FeS + HCO_3^-$ was calculated as $E_{298K} = 0.291 + 0.0074\log(a_{SO_4^{2-}}/a_{HCO_3^-}) - 0.0666$ pH. Approximate activities of $a_{HCO_3^-} = 10^{-2}$, and $a_{SO_4^{2-}} = 10^{-2.5}$ in the seawater system were assumed. For $\Delta_f G^0$ values underlying redox potential calculations, and for estimation of activities see Supplemental material. The free corrosion potential is between the equilibrium potentials of the coupled half-reactions.

completely covered with black precipitate, there was already a pronounced difference between the cathodic currents before and after glutaraldehyde treatment, viz. obvious biological cathodic activity. After eight days, the cathodic activity of strain IS4 was even more pronounced (Fig. 4b), indicating higher bacterial activity due to further increased cell numbers. However, the voltammogramms did not increase continuously, but rather exhibited irregularities. Apparently, the electrochemically stimulated bacterial sulfate reduction (Faradaic process) was overlaid by non-Faradaic processes, e.g. charging of capacitances in the porous semiconductive crust [22,34]; such non-Faradaic processes were indicated by supplementary potentiodynamic experiments with different sweep rates (Fig. S4). Additionally, glutaraldehyde-treated electrodes with strain IS4 (Fig. 4a, day 5 and 8) showed some residual activity with respect to the cathodic current in comparison to the electrokinetic behavior at the beginning (day 0) or to the electrodes incubated with control strain HS3. Therefore, residual metabolic activity (including the ability for enzymatic H_2 production [19,25]) of cells not fully inactivated due to deep burial in the crust cannot be excluded. Neither can some abiotic cathodic catalysis of the thick sulfidic precipitate formed by strain IS4 be excluded at this stage. A certain catalytic effect of ferrous sulfide in cathodic proton reduction could indeed be shown in a purely abiotic experiment. When a sterile, blank iron electrode was allowed to react with 1 mM dissolved sulfide (a concentration also produced by strain IS4 after eight days), the cathodic current increased slightly at a given ΔE (Fig. 5).

The electrode colonized by strain IS4 showed an increased current density at potentials down to $\Delta E = -150$ mV (Fig. 4a). If ΔE was shifted by more than −150 mV, the voltammogramms of the electrodes from different incubation periods approached each other and showed an exponential increase, as characteristic for abiotic H_2 evolution. As all enzymatic and living systems, the colonizing sulfate-reducing cells have their maximum (or saturation) activity (known as v_{max}) which is approached while the abiotic reaction is increasingly coming into play as the electrode potential deviates further from E_{corr}.

In conclusion, LSV measurements show that acceleration of cathodic reaction is largely a direct biological effect. This strongly supports the model of direct biological electron uptake by specialized lithotrophic SRB such as strain IS4 through an electroconductive, sulfidic corrosion layer. Furthermore, the absence of any cathodic stimulation by hydrogen-consuming strain HS3 (Fig. 4b) clearly challenges the classical 'cathodic depolarization theory', i.e. accelerated corrosion due to microbial H_2 uptake.

3.3. Electrical impedance spectroscopy of corrosion crust

Based on X-ray microanalyses, the measured conductivity of the corrosion crust formed by strain IS4 was attributed to the presence of FeS, which is a long-known semiconductor [19]. On the other hand, one may envisage that strain IS4 directs crust mineralization such that FeS within the carbonaceous crust assumes particularly conductive forms that do not occur in FeS–$FeCO_3$ co-precipitated unspecifically during mere consumption of 'cathodic' hydrogen. However, the conductivity of the crust formed by strain IS4 could not be compared to that of unspecific co-precipitate because the available quantities of the latter were insufficient for the previous

methodological approach [19]. Here, electrochemical impedance spectroscopy (EIS) was employed as a method suitable for small quantities to investigate possible differences with respect to the electronic properties of the precipitates formed on iron wires by the corrosive and non-corrosive strain.

EIS measurements indicated only a single time constant, irrespective of the bacterial strain and incubation time (Fig. S5). The data were fitted to a basic, Randles cell-like equivalent circuit (Fig. 6a). More elaborated equivalent circuits including for example several time constants (representing biofilms or passive layers) or Warburg impedances did not result in better fitting of the spectra. Whilst the fitting routine was more simplistic, the calculated results resemble previous findings [32,36,37].

An initial steep increase (up to around 45 $k\Omega\,cm^2$) in the polarization resistance (R_p) of sterile electrodes revealed the formation of a non-conductive, passive layer, probably consisting of iron carbonates and hydroxides (Fig 6b). Incubation of the iron wire with SRB decreased the resistance upon onset of sulfate reduction, indicating conversion of the initial layer to semiconductive (less than 1 $k\Omega\,cm^2$) sulfides. Conversion to iron sulfides and their further deposition was much faster and more pronounced in cultures of corrosive strain IS4 than in cultures of the non-corrosive strain HS3. The capacitance (C_d) of the formed layers was estimated using a constant phase element (CPE). In sterile incubations C_d remained at low values of around 0.1 to 0.3 $mF\,cm^{-2}$, implying the absence of charge mediation due to the non-conductive layer (Fig. 6c). In both SRB cultures C_d increased during incubation, approaching 4 $mF\,cm^{-2}$ in culture HS3 and exceeding 40 $mF\,cm^{-2}$ in culture IS4, indicating establishment of a conductive layer, most likely ferrous sulfide, that allows charging.

In conclusion, EIS allowed some insights into the conductive and dielectric properties of the precipitate formed on the iron due to the microbial metabolism. However, according to the present measurements, there is no evidence that the corrosive bacterium directs the co-deposition of FeS and $FeCO_3$ in a particular manner so that the precipitate is particularly advantageous for conducting electrons. We thus conclude that electrical conductivity of co-precipitated FeS and $FeCO_3$ is determined by their ratio and chemical conditions of precipitation rather than by biological factors.

4. Conclusions

Anaerobic microbial corrosion of iron in technical settings (*in situ*) represents a complexity of processes which are due to chemical effects of metabolites as well as to more intimate physiological microbe-metal interactions. Causal understanding of biocorrosion requires an experimental dissection into individual processes and their study under controlled conditions ('reductionistic' approach). A previously established approach, the (lithotrophic) use of metallic iron as the only electron donor for sulfate reduction and gain of energy for growth [19], was proven appropriate for gaining first insights into a supposedly central mechanism, the direct uptake of electrons from the metal through a semiconductive corrosion crust. A simplified microbiological model of such electron uptake is shown in Fig. 7A. The attached cells reduce sulfate (upon transport into the cell and activation to adenosine 5′-phosphosulfate) with electrons derived from iron via conductive ferrous sulfide and redox-active, cell-associated proteins (e.g. cytochromes). The particular proteins involved in the presently studied corrosive bacterium are unknown. Organic structures envisaged as mediators of electron flow into or out of living cells in contact with inorganic redox-active substances have been studied in other types of microorganisms [38–40]. Fig. 7B summarizes coupled electrochemical half-reactions that are relevant in the presently studied iron-seawater system. Even though effective microbial consumption of H_2 (g) to a fugacity of $a_{H_2} = 10^{-5}$, which is realistic under natural conditions [41,42], would significantly favor H^+-ion reduction from a purely thermodynamic point of view, direct electron consumption is kinetically favored. In conclusion, the present electrokinetic measurements corroborate the previously proposed model of a fast, biologically mediated by-pass of the slow reduction of H^+-ions to free H_2:

1. *D. corrodens* (tentative name) a representative of specially adapted, highly corrosive SRB, increases the free corrosion potential toward less negative values and enhances the current density at a given electrode potential.
2. *Desulfovibrio* sp. strain HS3, which resembles 'conventional' sulfate reducing bacteria, efficiently utilizes molecular hydrogen, including that formed on iron in water, but neither influences the free corrosion potential nor the current density at a given potential. Hydrogen consumption is thus not a decisive factor in microbial corrosion.
3. There is no indication for significant catalytic enhancement of the abiotic cathodic proton reduction to hydrogen by deposited ferrous sulfide crusts.
4. Rather, deposited ferrous sulfide as a semiconductor plays a significant role in anaerobic corrosion by mediating electron flow from the metal to the cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.corsci.2012.09.006.

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