Iron corrosion by novel anaerobic microorganisms

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Corrosion of iron presents a serious economic problem. Whereas aerobic corrosion is a chemical process[1], anaerobic corrosion is frequently linked to the activity of sulphate-reducing bacteria (SRB)[2–6]. SRB are supposed to act upon iron primarily by produced hydrogen sulphide as a corrosive agent[3,5,7] and by consumption of 'cathodic hydrogen' formed on iron in contact with water[2–6,8]. Among SRB, *Desulfovibrio* species—with their capacity to consume hydrogen effectively—are conventionally regarded as the main culprits of anaerobic corrosion[2–6,8–10]; however, the underlying mechanisms are complex and insufficiently understood. Here we describe novel marine, corrosive types of SRB obtained via an isolation approach with metallic iron as the only electron donor. In particular, a *Desulfobacterium*-like isolate reduced sulphate with metallic iron much faster than conventional hydrogen-scavenging *Desulfovibrio* species, suggesting that the novel surface-attached cell type obtained electrons from metallic iron in a more direct manner than via free hydrogen. Similarly, a newly isolated *Methanobacterium*-like archaeon produced methane with iron faster than do known hydrogen-using methanogens, again suggesting a more direct access to electrons from iron than via hydrogen consumption.

Some 10% of all corrosion damages to metals and non-metals may result from microbial activities[11]. A significant process in this respect is the anaerobic corrosion of iron or steel, for instance in oil technology[3,6,11]. The primary dissolution ($Fe \rightleftarrows Fe^{2+} + 2e^-$; $E^0 = -0.44\,V$) can in principle be driven by numerous oxidants. In oxic humid surroundings, oxidation by O_2 ($E^0_{pH7} = +0.82\,V$) yields rust[1]. In anoxic surroundings, H^+ ions from water yield H_2 ($E^0_{pH7} = -0.41\,V$). Of the sequential steps ($2e^- + 2H^+ \rightarrow 2H_{(adsorbed)} \rightarrow H_{2(adsorbed)} \rightarrow H_{2(aqueous)}$), the combination of the H atoms is presumably rate-limiting[12] and is a main reason for the slowness of iron oxidation in anoxic sterile water ($Fe + 2H_2O \rightarrow Fe^{2+} + H_2 + 2HO^-$). However, sulphate-reducing bacteria (SRB) promote the anaerobic oxidation of iron[2–6]. Their activity often occurs in biofilms and tends to pit the iron[2,3,13]. An indirect and a direct corrosion mechanism may be distinguished[3,5]: these may occur simultaneously at different extents, depending on the load of waters with biodegradable organic compounds.

The indirect mechanism is a chemical attack by hydrogen sulphide ($Fe + H_2S \rightarrow FeS + H_2$) which is faster than that by water[3,5,7] and also promotes so-called hydrogen embrittlement of the metal[1,12] (see also http://www.corrosion-doctors.org). Because SRB commonly use organic compounds (shown here as $[CH_2O]$) and often also H_2 for sulphate reduction, the net reaction of indirect corrosion (for complete carbon oxidation by SRB communities) can be written as:

$$2[CH_2O] + 1\tfrac{1}{3}Fe + 1\tfrac{1}{3}\,SO_4^{2-} + \tfrac{2}{3}H^+ \rightarrow 2HCO_3^- + 1\tfrac{1}{3}FeS + 1\tfrac{1}{3}H_2O \quad (1)$$

In the direct mechanism according to the depolarization theory (see Supplementary Information)[8], SRB are supposed to stimulate corrosion by scavenging 'cathodic hydrogen' or a 'hydrogen film'

on water-exposed iron (often written with unspecified hydrogen as $8[H] + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_2O$). The resulting net reaction of direct corrosion is:

$$4Fe + SO_4^{2-} + 4H_2O \rightarrow FeS + 3Fe^{2+} + 8HO^- \quad (2)$$

In addition to FeS, also $Fe(OH)_2$ or $FeCO_3$ can precipitate. The direct corrosion mechanism is commonly attributed to *Desulfovibrio* species[3–6,8–10,13], the best-studied SRB, and to their efficient H_2 utilization[14,15]. Indeed, stimulating effects of *Desulfovibrio* cells on the current via iron cathodes have been observed[9,10]. On the other hand, stimulation of iron oxidation due to consumption of chemically formed H_2 has been questioned[5,6,16,17]; for instance, H_2 did not inhibit its own formation on iron in sterile water[6]. It is true that *Desulfovibrio* species[4,10,18] and also methanogenic archaea[19,20] formed sulphide or methane, respectively, with metallic iron in growth media; however, this was apparently due to secondary consumption of chemically formed H_2 without stimulation of corrosion[6,20].

To search for possibly yet undetected SRB with potential for direct corrosion (equation (2)), we established enrichment cultures with iron specimens as the only electron donor and marine sediment as inoculum. Iron has been used as a reductant in a former enrichment technique[21], but has not been reported to yield cultures other than *Desulfovibrio* species that grow with organic substrates or H_2. For comparison, we enriched parallel cultures with H_2 instead of iron. Within two weeks, sulphate reduction in cultures with iron exceeded the endogenous sulphate reduction with sediment alone in iron- and H_2-free controls (4 mM versus 2 mM). Consecutive subcultures with iron were inoculated with a part of the previous iron specimens. As carbon sources, cultures contained either CO_2 alone, or CO_2 plus acetate (1 mM). Sulphate reduction in subcultures became faster, and black layers of ferrous sulphide became visible. Microscopy revealed only a few free-living cells. In contrast, the enrichment culture with H_2 yielded abundant free-living cells.

Two representative strains, IS4 and IS5, were isolated from iron-grown sulphate-reducing enrichments (without or with acetate, respectively). A third strain, HS2, was isolated from the H_2-grown enrichment (with acetate). Strain IS4 is rod-shaped (Fig. 1a–c) and affiliates with *Desulfobacterium* (Fig. 2), whereas the other two strains are comma-shaped (Supplementary Fig. 1) and represent *Desulfovibrio* species. All three strains also grew by sulphate reduction with lactate or H_2. With H_2 and CO_2, strain IS4 did not depend on an organic carbon source, whereas strains IS5 and HS2 needed acetate (for cell synthesis[14]). With lactate or H_2, strain IS4 exhibited much slower growth (doubling time, $\geq$2 days) than strains IS5, HS2 and the authenticated species, *Desulfovibrio salexigens* and *D. vulgaris* (doubling time, $<$1 day).

Probing of the enrichment culture with a fluorescent 16S ribosomal RNA-targeted oligonucleotide specific for strain IS4 revealed high numbers of precipitate-associated cells with shape similar to that of strain IS4 (Fig. 1d, e); they represented the majority of the detectable cells. A common oligonucleotide probe for *Desulfovibrio* species[22] did not hybridize.

We also enriched marine methanogenic microorganisms with metallic iron in low-sulphate medium. Iron-dependent methane production first became obvious after 20 days. From the fourth subculture, a methanogenic strain, IM1, was isolated. Attempts to retrieve a 16S rRNA gene sequence from strain IM1 yielded only a short fragment (1,000 bp) revealing an affiliation with *Methanobacterium* and *Methanobrevibacter* (Supplementary Fig. 2). In the absence of metallic iron, strain IM1 grew slowly with $H_2 + CO_2$ if the pH was above 7.5.

For direct measurement of corrosiveness, we followed sulphate reduction or methanogenesis with metallic iron as the only electron donor by the newly isolated as well as by authenticated SRB and methanogenic archaea, respectively (Fig. 3a, b). The authenticated species are known hydrogen utilizers. Sulphate reduction to sulphide by strain IS4 with iron was fast, much as in the enrichment culture. Sulphate reduction gradually slowed down, but became faster again if fresh iron was added (not shown), suggesting that formed crusts (Fig. 1a) act as process barriers. Sulphate reduction by strain IS5 was slower than by strain IS4, but faster than by strain HS2 and the authenticated *D. salexigens* and *D. vulgaris*. Sulphate reduction rates of the latter three were in agreement with a secondary consumption of chemically formed H_2 (1 mol sulphate requiring 4 mol H_2). Methanogenesis with iron was also more pronounced with the new strain IM1 than with the authenticated *Methanococcus maripaludis* (Fig. 3b), *Methanogenium organophilum* and *Methanosarcina mazei* (not shown). The rate of methanogenesis with iron by the authenticated species was again in accordance with a secondary consumption of chemically formed H_2 (1 mol CH_4 requiring 4 mol H_2).

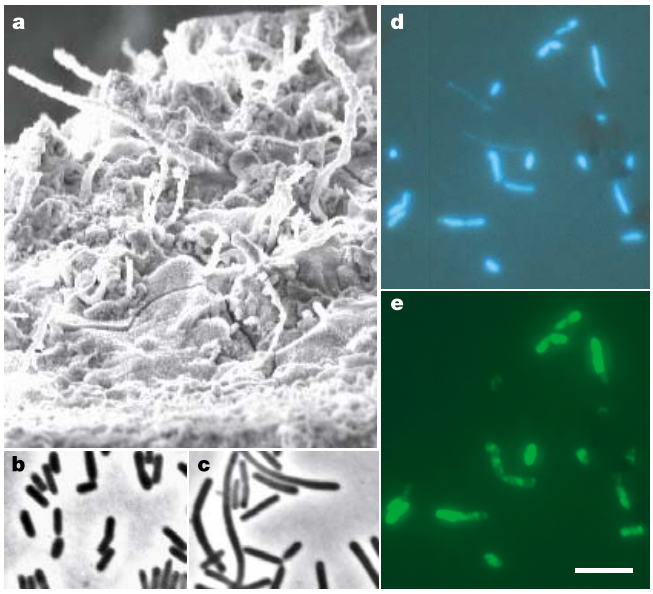

Figure 1 Microscopy of cultures. **a**, Scanning electron micrograph of an iron coupon incubated with strain IS4 for seven weeks. Filamentous cells are embedded in precipitated $FeCO_3$ and FeS. **b**, Phase contrast micrograph of viable cells of strain IS4 grown for 1 week with lactate. **c**, Cells in the same culture after three weeks. **d**, General (DAPI) staining of cells in dark precipitate in an enrichment culture with metallic iron. **e**, The same section with visualization of fluorescent (tyramide-fluorescein) staining via a specific oligonucleotide probe for strain IS4. Bar, 10 μm (applicable to all panels).

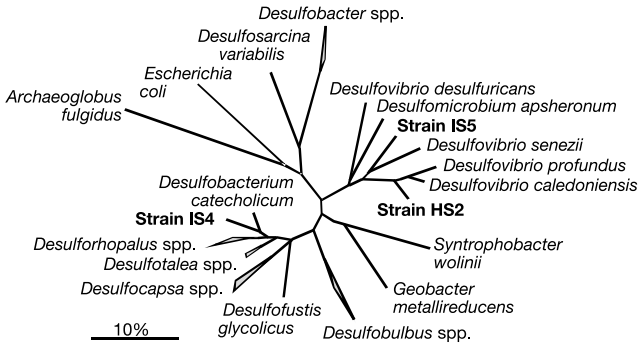

Figure 2 Phylogenetic relationships (based on 16S rRNA gene sequences) of new isolates of sulphate-reducing bacteria from enrichment cultures with metallic iron (strains IS4 and IS5) or hydrogen (strain HS2) and sulphate. Bar indicates 10% estimated sequence divergence.

Because the speed of sulphate reduction by strains IS4 and IS5 and of methanogenesis by strain IM1 with iron cannot be explained by mere consumption of the chemically formed hydrogen, these organisms must obtain reducing equivalents more efficiently. In cultures of strain IS4 with iron, H_2 accumulated to high levels (Fig. 3c) and hence cannot be the rate-limiting intermediate; this was not observed in incubations with strains IS5, HS2 and the authenticated *Desulfovibrio* species. An efficient use of metallic iron for sulphate reduction (or methanogenesis) would be possible by an electron uptake via a cell-surface-associated redox-active component, a principle known from aerobic iron(II)-oxidizing bacteria[23]. The inverse process, a delivery of electrons (from organic electron donors) by cells to external solid or dissolved matter occurs in iron(III)-reducing bacteria[24]. Following hydrogen formation assays with cell fractions of *D. vulgaris*, a cytochrome in the outer membrane has been suggested to participate in iron corrosion; an electron flow to the sulphate reduction enzymes via two types of periplasmic hydrogenase and H_2 as a direct intermediate was proposed, according to: Fe → cytochrome → hydrogenase(1) → H_2 → hydrogenase(2) → electron transport system → sulphate reduction enzymes[17] (arrows indicate electron flow). In our study, however, the same *D. vulgaris* did not reveal a corrosive potential like strains IS4 and IS5. Nevertheless, the assumption of a cell-surface-associated redox component is an obvious hypothesis to explain how the new isolates can circumvent the slow chemical formation of H_2 (ref. 12) to obtain their reducing equivalents. The pronounced H_2 formation during growth of strain IS4 with metallic iron does not necessarily indicate that H_2 is a direct biochemical intermediate connecting two hydrogenases[17]. H_2 may just as well be formed via a branch according to:

$$\begin{array}{c} \mathrm{Fe} \rightarrow \text{electron transport system} \rightarrow \text{sulphate reduction enzymes} \\ \uparrow\downarrow \\ \text{hydrogenase} \rightleftarrows \mathrm{H_2} \end{array} \qquad (3)$$

as a result of an imbalance between electron donation by fresh iron and electron consumption by sulphate reduction (see also Supplementary Fig. 3). The hydrogenase may otherwise function in growth with external hydrogen. Detailed models of electron flow are at present not possible because knowledge of the topology and function of redox proteins in various SRB[14] (and methanogens[25]) is insufficient.

The abundance of cells resembling strain IS4 in our enrichment culture with iron and the effective iron-dependent sulphate reduction by this strain suggests a thus-far overlooked involvement of such or physiologically similar SRB in anaerobic corrosion. Proof of this assumption requires the examination of technical plants with corroding iron or steel. The natural significance of the capacity for using metallic iron as electron donor is unknown. Apart from rare meteorites, metallic iron is a technical product and is thus a very 'recent' growth substrate. One may speculate that iron-using anaerobes can also obtain electrons in cell contact with certain microorganisms. □

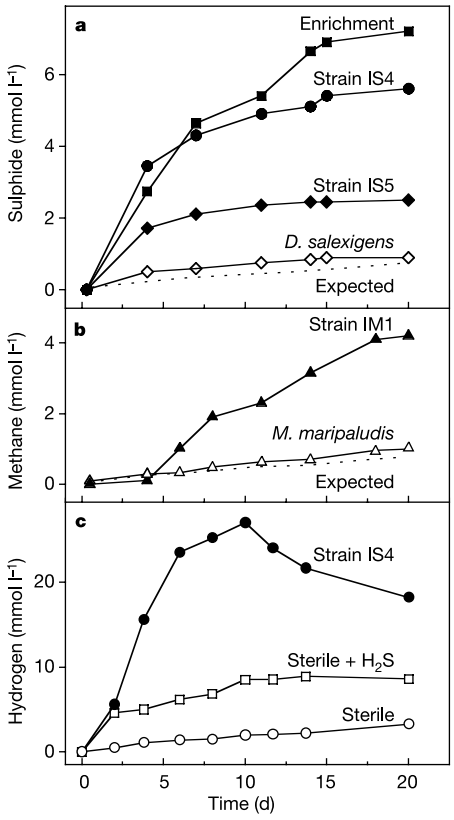

Figure 3 Incubation experiments with iron granules (30 g in 150 ml medium) as sole electron donor. **a**, Sulphide formation (via sulphate consumption) by strains IS4, IS5 and *Desulfovibrio salexigens*, and the original enrichment culture. **b** Methane formation by strain IM1 and *Methanococcus maripaludis*. **c**, Hydrogen formation by strain IS4 and in sterile iron incubations without or with hydrogen sulphide (4 mM). In the latter, hydrogen production became slower after binding of free sulphide as FeS. **a**, **b**, Sulphate reduction or methanogenesis expected from mere consumption of chemically formed hydrogen is indicated by dotted lines.

Methods

Enrichment, isolation and cultivation

Marine sediment was collected near Wilhelmshaven, North Sea. Cultures were grown at 28 °C in anoxic seawater (marine organisms) or freshwater (*D. vulgaris*) medium with 28 mM sulphate (0.1 mM for methanogens) under $N_2 + CO_2$ (90/10, vol./vol.)[15]. If indicated, 1 mM sodium acetate was added as organic carbon source. Iron granules (99.8% Fe, size 2 mm, 20 g per 100 ml) or mild steel coupons (1 mm thickness, fitted to tubes or bottles) were added as the electron source. For subculturing, 10% of the culture liquid and part of the iron specimens were transferred every seven weeks. Parallel enrichment cultures were carried out with $H_2 + CO_2$ (+1 mM acetate) without metallic iron.

For isolation of enriched organisms, precipitates from the iron surface were homogenized anaerobically and sequentially diluted in medium with iron granules. The highest dilutions showing sulphate reduction or methanogenesis were again diluted. SRB were finally diluted in anoxic agar[15] with a mixture of lactate, propionate, butyrate, pyruvate, ethanol (each 2 mM) and hydrogen. Colonies were transferred to liquid medium.

Authenticated strains were from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Chemical analyses

Sulphate was determined micro-gravimetrically as $BaSO_4$ precipitated from 1-ml samples with the same volume of 0.2 M $BaCl_2$ and 0.2 M HCl. CH_4 and H_2 were quantified on a gas chromatograph with a flame ionization or thermal conductivity detector, respectively. Minerals on the iron surface were analysed by X-ray photoelectron spectroscopy.

Analyses of 16S rRNA genes

DNA was extracted with the DNeasy Tissue kit (Qiagen). Nearly full-length (1,500 bp) 16S rRNA gene sequences from SRB were amplified using general bacterial primers[26]. A partial (about 1,000 bp) 16S rRNA gene sequence of the methanogenic isolate was amplified with archaeal primers[27]. Sequences were analysed using the ARB program[28], and a phylogenetic tree was constructed via maximum-parsimony, neighbour-joining and maximum-likelihood analyses. The methanogenic strain IM1 was positioned in the tree according to parsimony criteria without affecting the overall topology.

Specific cell hybridization and unspecific staining

Precipitates scratched from corroded iron surfaces were fixed for 12 h at 4 °C in 4% formaldehyde, washed twice with PBS (10 mM sodium phosphate, pH 7; 130 mM NaCl), stored in PBS-ethanol (1:1) at −20 °C, and collected on polycarbonate filters (0.2 μm pores; Millipore). A horseradish peroxidase-labelled probe (5′-CTCCTCCTGCTGCAGTAGCT-3′) was specifically designed and synthesized (Thermohybaid) for strain IS4. After hybridization at 35 °C in the presence of 55% (vol./vol.) formamide and washing, the probe was reacted with tyramide-fluorescein[29]. DAPI (4′,6-diamidino-2-phenylindole) was used for general cell staining.

Remarks on redox potentials and equations

Indicated redox potentials are versus standard hydrogen electrode ($E^0 = 0$ V). The Fe^{2+}/Fe redox potential in cultures and sea water is much more negative than under standard conditions (−0.44 V); whereas Fe remains in standard state, salts and

precipitation as sulphide and carbonate decrease the Fe^{2+} activity[5] such that redox potentials around -0.53 V are realistic. Proton reduction to H_2 ($E^0_{pH7} = -0.41$ V) on metallic iron in such surroundings would thus be far from the thermodynamic equilibrium.

Equation (1) is based on the equations for sulphate reduction with the organic compound ($2[CH_2O] + SO_4^{2-} \rightarrow 2HCO_3^- + H_2S$) and hydrogen ($4H_2 + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_2O$). If SRB reduce 1 mol SO_4^{2-} with an organic compound to 1 mol H_2S, the latter yields 1 mol H_2 upon chemical reaction with Fe to FeS. Use of H_2 for further sulphate reduction yields $^1/_4$ mol H_2S which leads to $^1/_4$ mol H_2. Continuation ad infinitum leads to a total of $1^1/_3$ (sum of infinite row $1 + {}^1/_4 + {}^1/_{16}$ etc.) mol H_2S that attacks the iron. (For other remarks on reactions in corrosion see Supplementary Information.)

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Correspondence and requests for materials should be addressed to F.W. (fwiddel@mpi-bremen.de). The nucleotide sequences have been deposited at EMBL GenBank under accession numbers AY274444 (strain HS2) AY274449 (strain IS5), AY274450 (strain IS4) and AY274451 (strain IM1).