

Microbiological study of the anaerobic corrosion of iron

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Dinh Thuy Hang

aus Hanoi

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1. Gutachter: Prof. Dr. Friedrich Widdel, Universität Bremen
2. Gutachter: Prof. Dr. Heribert Cypionka, Universität Oldenburg

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To my parents

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List of abbreviations

(Very common abbreviations and units are not listed)

atm	atmosphere (= 101 kPa)
A+T	Adenine and thymine
bp	Base pair
BSA	Bovine serum albumin
CM	Cytoplasmic membrane
d	day
DAPI	4',6-diamidino-2-phenylindole
Dbm212, DSV698	Fluorescent oligonucleotide probes specific for <i>Desulfobacterium</i> and <i>Desulfovibrio</i> species, respectively
DGGE	Denaturing gradient gel electrophoresis
DSMZ	Deutsch Sammlung von Mikroorganismen und Zellkulturen GmbH
∅	Diameter
EcoRI	A restriction enzyme
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolymeric substance
EUB338	Fluorescent oligonucleotide probe specific for the bacterial domain
FID	Flame ionization detector
FISH	Fluorescence <i>in situ</i> hybridization
G+C	Guanine and cytosine
H.A.Yellow	Commercial name of bisbenzimidazole-polyethylene glycol
HPLC	High performance liquid chromatography
[H]	Reducing equivalent
H ₂ ase	Hydrogenase
Me	Metal
MIC	Microbially influenced (induced) corrosion
OD	Optical density
PBS	Phosphate saline buffer
PCR	Polymerase chain reaction
PP _i	Pyrophosphate (diphosphate)
ppm	parts per million
ppmv	parts per million (referring to volume)

rpm	rotations per minute
rRNA	Ribosomal ribonucleic acid
SEM	Scanning electron microscopy
sp	Species
SRB	Sulfate-reducing bacteria
vol/vol	volume/volume
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TCD	Temperature conductivity detector
t_d	Doubling time
XPS	X-ray photoelectron spectroscopy

Summary

Anaerobic corrosion causes economically significant damages. The industrial branches that suffer from MIC most severely include the nuclear and fuel electric power-generating sectors as well as the oil industry. Despite of numerous investigations of anaerobic corrosion, the underlying mechanisms are insufficiently understood. In the present study, the anaerobic corrosion of iron was investigated in seawater under conditions of sulfate reduction and methanogenesis. Enrichment cultures of sulfate-reducing bacteria (SRB) and methanogenic archaea using metallic iron (Fe) as the only source of electrons were established with marine sediment samples as inocula. Further aspects that were subsequently investigated were the composition of the enriched microbial community, the key organisms involved in corrosion, and mechanistic aspects of the process at the iron surface.

1. Corrosion of iron by sulfate-reducing bacteria

a) For the first time, SRB were directly enriched with metallic iron (Fe) and sulfate as the only growth substrates in CO₂/bicarbonate-buffered medium. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments revealed characteristic bands affiliating with the *Desulfobacterium* and *Desulfovibrio* clusters (Deltaproteobacteria) in the iron-grown enrichment cultures. These bands were absent in enrichment cultures with H₂ as electron donor instead of iron (control experiments), and therefore were proposed to represent populations of SRB that play a crucial role in the process of iron corrosion. The *Desulfobacterium*-related population was detected in the iron-grown enrichment cultures with marine sediment from geographically distant sampling sites, indicating a possibly widespread type of iron-corroding sulfate-reducing bacterium.

b) A rod-shaped sulfate reducer, strain IS4, was isolated from the enrichment culture with iron. Cells of this strain exhibited a strong tendency to associate with the iron surface. Analysis of the nearly full length 16S rRNA gene sequence showed that strain IS4 is related to the genus *Desulfobacterium*, the closest relative being *Desulfobacterium catecholicum* (95% sequence similarity). However, physiological properties of strain IS4 differed significantly from those of *Desulfobacterium catecholicum*. Strain IS4 is regarded as a new species of the genus *Desulfobacterium* and the name *Desulfobacterium corrodens* is proposed.

c) Fluorescent *in situ* hybridization using a specific, newly designed oligonucleotide probe revealed that the new type of sulfate-reducing bacterium represented by strain IS4 predominated in the bacterial community enriched with iron.

d) Strain IS4 used only iron, H₂, lactate and pyruvate for the reduction of sulfate. When iron served as electron donor, strain IS4 reduced sulfate more rapidly than *Desulfovibrio* species; in contrast, with H₂ or lactate this strain reduced sulfate remarkably slower than *Desulfovibrio* species. The rate of sulfate reduction with iron by strain IS4 was similar to that by the enrichment culture from which the strain had been isolated. Significant accumulation of H₂ was observed during the early growth phase of strain IS4 with iron. It was calculated from the rate and stoichiometry of sulfate reduction with iron that strain IS4 derived electrons (reducing equivalents) from the iron at a fivefold higher rate than could be achieved via the chemically formed H₂ (according to $\text{Fe} + 2 \text{H}^+ \rightarrow \text{Fe}^{2+} + \text{H}_2$). It is, therefore, assumed that electrons from iron can be taken up by strain IS4 in a more direct manner than via hydrogen.

e) In addition, a *Desulfovibrio* species, strain IS5, with close relationship to *Desulfovibrio senezii* (92% sequence similarity) was isolated from the iron-grown enrichment culture with CO₂ and acetate as carbon source. Strain IS5 reduced sulfate with iron at a higher rate than other *Desulfovibrio* species obtained from enrichment cultures with H₂ or a culture collection, but at a lower rate than strain IS4. The rate and stoichiometry of sulfate reduction with iron by strain IS5 suggested that in addition to the utilization of chemically formed H₂, the strain also gained electrons directly from the iron. Due to significant phylogenetic distance to its closest cultivated relative, *Desulfovibrio senezii* (92% similarity in 16S rRNA gene sequence), strain IS5 is regarded as a new species of the genus *Desulfovibrio* and the name *Desulfovibrio ferrophilus* is proposed.

2. Corrosion of iron by methanogenic archaea

a) Methane production from metallic iron and CO₂ was demonstrated in a marine enrichment culture. Sequence analysis of PCR amplified fragments of the 16S rRNA gene revealed that this enrichment culture contained one dominant population with a sequence similarity of 96% to *Methanobacterium* spp. and *Methanobrevibacter* spp.

b) A pure methanogenic culture designated strain IM1 was isolated from the enrichment culture via liquid dilution series in mineral medium containing iron as the only electron donor. Sequence analysis of a 1000 bp fragment of the 16S rRNA gene of strain IM1 revealed a close

relationship to *Methanobacterium* spp. and *Methanobrevibacter* spp. (96% sequence similarity to each genus). Full-length sequencing of the 16S rRNA gene of strain IM1 could not be achieved by using the available primer sets specific for the archaeal domain, indicating that the isolate represents a hitherto unknown line of descent within the methanogenic Archaea.

c) It was calculated that the rate of methane production by strain IM1 with iron and CO₂ was significantly higher than that expected with chemically formed H₂ (according to $\text{Fe} + 2 \text{H}^+ \rightarrow \text{Fe}^{2+} + \text{H}_2$). AT pH above 7.5 strain IM1 could grow slowly with H₂, at a rate significantly slower than H₂-utilizing methanogens such as *Metanococcus maripaludis* and *Methanogenium organophilum*. These results provide further evidence for an electron transfer from iron to the cells without involvement of hydrogen as an intermediate.

3. An alternative mechanism of cathodic depolarization

The corrosive SRB or methanogens are supposed to gain electrons directly from metallic iron for sulfate or CO₂ reduction, respectively, in intimate contact with the iron surface. An involvement of certain cell components in the flow of electrons through the outer cell barrier has to be postulated. Reduction of protons to form H₂ may also occur as a side reaction that involves periplasmic hydrogenases. It is supposed that high corrosion rates as detected under field conditions are mostly the result of a combination of two processes (i) the direct electron uptake from iron by attached cells and (ii) the microbial production of corrosive H₂S.

Part I. Biocorrosion of iron: Overview of the literature and results of the present study

A. Overview of the literature

1. Introductory remarks on economic significance and principal reactions during corrosion

Iron is the least expensive and most widely used metal in technology. According to reports by the International Iron and Steel Institute (IISI; <http://www.worldsteel.org>), the total steel production and consumption over the world has been steadily increasing, reaching 845 million metric tons in 2001. Mild steel (carbon steel) is iron containing 0.4 to 1.7% carbon, whereas stainless steel contains substantial proportions of nonferrous metals such as chromium or nickel as protective additives. Iron as a base metal is usually unstable without protection and easily undergoes corrosion in aqueous environments. Corrosion has been defined as the “destructive attack of a metal by chemical or electrochemical reactions” (Uhlig, 1985). In aqueous environments, iron materials are corroded not only by purely chemical or electrochemical reactions but also by metabolic activities of microorganisms in a process termed microbially influenced (or induced) corrosion (MIC).

Corrosion of iron materials causes vast economic damages and is, therefore, of great concern. According to recent investigations, damages due to material corrosion in the United States cause annual costs of 276×10^9 \$ in many fields of the industry (Fig. 1) (Koch et al., 2002). Other studies undertaken in several countries including the United Kingdom, Japan, Germany, Sweden and Australia revealed that the annual costs due to corrosion damages ranged from 1 to 5% of the gross national product (GNP) of each nation (www.corrosion-doctors.org; assessed 20.08.02).

Among the various corrosion processes, the microbially influenced corrosion (MIC) of materials is reported to account for up to 50% of the damage costs (Hamilton, 1985; Tiller, 1988; Ross et al., 1993; Fleming, 1996). The industries that are suffering loss due to MIC most severely include the nuclear and fuel electric power generating sectors, pipelines, oil fields and offshore industry (Dowling and Guezennec, 1997). In some municipal systems such as drinking water distribution systems, high rates of MIC not only cause significant losses to the economy, but also directly affect the public health (Volk et al., 2000).

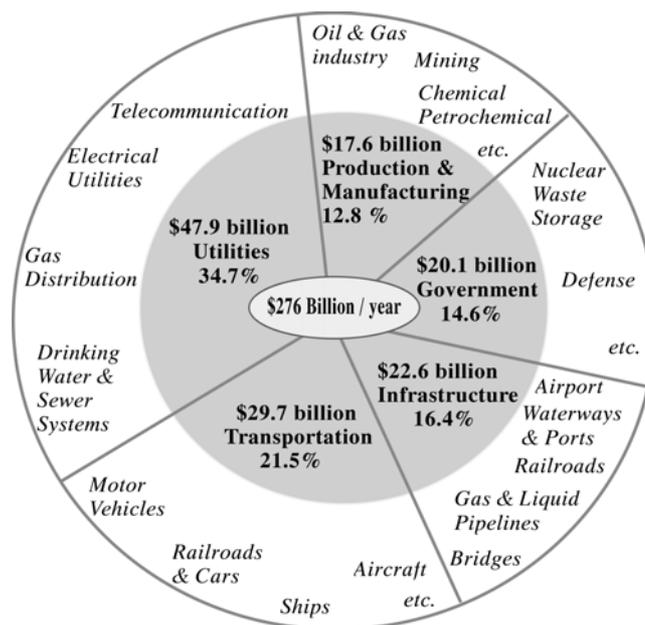


FIG. 1. Costs caused by corrosion in the USA (www.corrosioncost.com; assessed 20.08.2002)

If a metal comes into contact with water, positive metal ions are released into the solution and leave free electrons on the metal:



The reaction shifts to the right if the liberated electrons are continuously removed, resulting in a net dissolution of the metal. Free electrons cannot be released as such into the medium; usually they can be consumed by reactions with oxidizing substances from the aqueous phase at the metal-water boundary. Such electron acceptors might be oxygen, protons, undissociated weak acids or water (Uhlig, 1985). Areas on the metal where metal dissolution or electron uptake reactions occur are termed anodic and cathodic sites, respectively. The accumulation of products of the cathodic and anodic reactions at the metal-water interface tends to slow down the rate of corrosion. This process is termed polarization; it may be broken down if the corrosion products are removed, leading to depolarization and consequently to continuous corrosion.

Microorganisms are able to depolarize both cathodic and anodic sites either directly by their metabolic activities or indirectly by excretion of chemically reactive products (Miller, 1981; Iverson, 1987; Widdel, 1992a). Such microorganisms are particularly corrosive as they grow in colonies or films attached to iron surface and thereby create local electrochemical

cells with highly stimulated reactions. As a result, corrosion by microorganisms often occurs as pitting, which is usually more severe than corrosion processes that are evenly distributed over the metal surface (Hamilton and Lee, 1995; Lee et al., 1995; Cord-Ruwisch, 2000). Under air, metal corrosion is linked with an attack of oxygen which is mainly chemical (Uhlig, 1985). On the other hand, metallic iron also undergoes severe corrosion in the absence of oxygen, sometimes even at higher rates than in the presence of oxygen (Lee et al., 1995). Such an anaerobic corrosion is mostly due to microbial activities (Miller, 1981; Hamilton, 1985; Iverson, 1987; Crolet, 1992). The most aggressive corrosion is usually observed in oxic-anoxic environments where both aerobic and anaerobic microorganisms develop (Lee et al., 1995; Videla, 2001).

2. Aerobic microbial corrosion

Aerobic microbial corrosion involves complex chemical and microbial processes due to metabolic activities of different groups of microorganisms. Usually, even in aerobic corrosion, oxygen concentration may be very low, for instance underneath microbial colonies or biofilms (Costerton et al., 1995; Santegoeds et al., 1999; De Beer and Stoodley, 2000). The anodic dissolution of Fe to Fe²⁺ preferentially takes place at such micro-oxic to anoxic sites, whereas electrons flow to the other sites where they can reduce molecular oxygen (Miller, 1981). The Fe²⁺ formed may be oxidized chemically or by iron-oxidizing bacteria to hydrates of ferric oxides that are deposited as rust on the metal surface (Nealson et al., 1983; Uhlig, 1985).

Pseudomonas species and other slime-forming bacteria are commonly found in connection with corrosion. They colonize the metal surface, thereby creating oxygen-free environments for anaerobic bacteria, especially sulfate reducers (Costerton et al., 1995; Flemming and Schaule, 1996; Vidella, 2001). Exopolymeric substances (EPS) excreted by these bacteria may contain organic acids and salts at high concentration which may stimulate metal deterioration (Gaylarde et al., 1988; Sand et al., 1996).

Some groups of aerobic bacteria produce strong inorganic acids and thus become very corrosive toward iron. The most significant group is the genus *Thiobacillus*, members of which produce sulfuric acid by oxidizing sulfur species (Kelly, 1989). *Thiobacillus thiooxydans* and *Thiobacillus ferrooxydans* are the most common representatives that have been reported to be involved in corrosion (Miller, 1981; Tributsch et al., 1998; Gu and Mitchell, 2000).

A third group of bacteria that may contribute to aerobic metal corrosion are “iron” bacteria, including the stalked bacteria of the genus *Gallionella* and the filamentous bacteria of the genera *Leptothrix*, *Clonothrix*, *Sphaerotilus*, *Crenothrix* and *Lieskeella* (Iverson, 1987; Mulder and Deinema, 1992; Ehrlich, 1996). Members of this group may gain energy from the oxidation of ferrous to ferric iron, or at least stimulate such a process which results in massive depositions of ferric hydroxide. As a consequence, condensed anoxic zones are formed and the metal surface is partitioned into small anodic sites exposing to large surround cathodic areas where electrons reduce the available oxygen (Little and Wagner, 1997; Rao et al., 2000; Starosvetsky et al., 2001). A formation of tubercles in iron steel is the common type of corrosion by these bacteria (Miller, 1981; Iverson, 1987; Gu and Mitchell, 2000; Starosvetsky et al., 2001).

Fungi and algae may be also involved in metal deterioration. In fuel and oil storage tanks, fungi species such as *Aspergillus*, *Penicillium* and *Fusarium* may grow on fuel components and produce carboxylic acids which corrode the iron (Iverson, 1987; Little and Wagner, 1997; Little et al., 2001). In the presence of light, algae can produce organic acids and decrease the pH in the environment, thereby favoring corrosion (Mara and Williams, 1972).

3. Anaerobic microbial corrosion

Iron and iron alloys also corrode severely in oxygen-free environments (Miller, 1981; Hamilton 1985; Widdel, 1992a; Cord-Ruwisch, 2000). Pipelines, offshore oil platforms and underground structures have been reported to be quite vulnerable to biological corrosion which is assumed to be mediated by different groups of microorganisms respiring with oxidized compounds such as sulfate, nitrate, ferric iron or carbon dioxide (Miller, 1981; Iverson 1987; Widdel, 1992a).

3.1 Anaerobic corrosion by sulfate-reducing bacteria (SRB)

Sulfate-reducing bacteria are proposed to be chiefly responsible for anaerobic corrosion, particularly in environments with high sulfate concentrations such as seawater (Cord-Ruwisch et al., 1987; Hamilton et al., 1988; Cord-Ruwisch, 1995; Hamilton, 1998a; 1998b). From a scientific point of view, the mechanistic aspects of the interaction between these organisms and iron are of special interest. The mechanism by which sulfate reducers accelerate metal

corrosion has attracted many investigators, but details of the process are still inadequately understood (Hamilton, 1985; Iverson, 1987; Widdel, 1992a; Cord-Ruwisch, 2000).

3.1.1 Physiology and phylogeny of SRB

Sulfate-reducing bacteria (SRB) are abundant in natural habitats such as marine and fresh water sediments or sludges and play a key role in the biogeochemical sulfur cycle (Jørgensen, 1983; Widdel, 1988; Fauque, 1995). SRB are obligately anaerobic bacteria that gain energy for growth by oxidizing organic compounds or H₂ with SO₄²⁻ being reduced to H₂S (Postgate, 1984; Barton and Tomei, 1995; Rabus et al., 2000). In comparison to oxygen respiration, sulfate respiration provides significantly less energy, for instance with acetate:



SRB therefore synthesize less cell mass per mol of an organic substrate oxidized than oxygen-respiring organisms (Widdel, 1988; Rabus et al., 2000).

Alternatively, several SRB may reduce nitrate, sulfite, thiosulfate or fumarate with organic compounds or H₂ to gain energy for growth (Widdel 1988; Cypionka, 1995; Rabus et al., 2000). Fermentation is usually observed with pyruvate and sometimes with lactate, malate, or fumarate (Thauer et al., 1977; Cypionka, 1995; Rabus et al., 2000). Although being strictly anaerobic, some sulfate reducers may tolerate oxygen at low concentrations or even can oxidize organic substrates or H₂ with oxygen as the terminal electron acceptor and couple this reaction to the formation of ATP (Dilling and Cypionka, 1990; Marschal et al., 1993; Cypionka, 2000). However, there is no convincing evidence so far for aerobic or microaerobic growth of SRB.

Phylogenetically, SRB may be divided into four major groups, as based on 16S rRNA gene sequence analyses. These are the Gram-negative mesophilic SRB of the *δ-Proteobacteria*, the Gram-positive spore-forming SRB, the deeply branching thermophilic SRB, and the thermophilic archaeal sulfate reducers (Fig. 2) (Stackebrandt et al., 1995; Castro, 2000, Rabus et al., 2000). The mesophilic SRB of the *δ-Proteobacteria* represent the largest group which presently comprises around 20 genera (Devereux et al., 1989; Widdel and Bak, 1992, Stackebrandt et al., 1995). The spore-forming genera *Desulfotomaculum* and *Desulfosporosinus* branch with other Gram-positive bacteria of low G+C content, whereas the thermophilic SRB *Thermodesulfo-bacterium* and *Thermodesulfovibrio* make up separate

branches distant from the mesophilic SRB (Stackebrandt et al., 1995; Rabus et al.; 2000). Archaeal sulfate reducers of the genus *Archaeoglobus* are related to methanogens and some extremophiles in the domain *Archaea* (Stetter et al., 1987; Stetter, 1996).

Most known species of SRB are mesophilic and grow optimally between 20 and 40 °C (Widdel, 1988). The genus of spore-forming *Desulfotomaculum* comprises some moderately thermophilic species that grow optimally between 55 and 65 °C (Nilsen et al., 1996a; 1996b). Species of the thermophilic genera *Thermodesulfobacterium* and *Thermodesulfovibrio* as well as archaeal sulfate reducers of the genus *Archaeoglobus* have been reported to grow optimally between 70 °C and 90 °C (Stetter et al., 1987; Burggraf et al., 1990; Widdel, 1992b; Stetter, 1996). SRB were also detected at relatively high numbers in permanently cold, arctic marine sediments with temperatures below 0 °C (Knoblauch et al., 1999b; Sahm et al., 1999). Psychrophilic isolates of sulfate reducers from such sediments showed the highest growth rate and sulfate-reduction rate at 7–10 °C (Knoblauch et al., 1999a, 1999c).

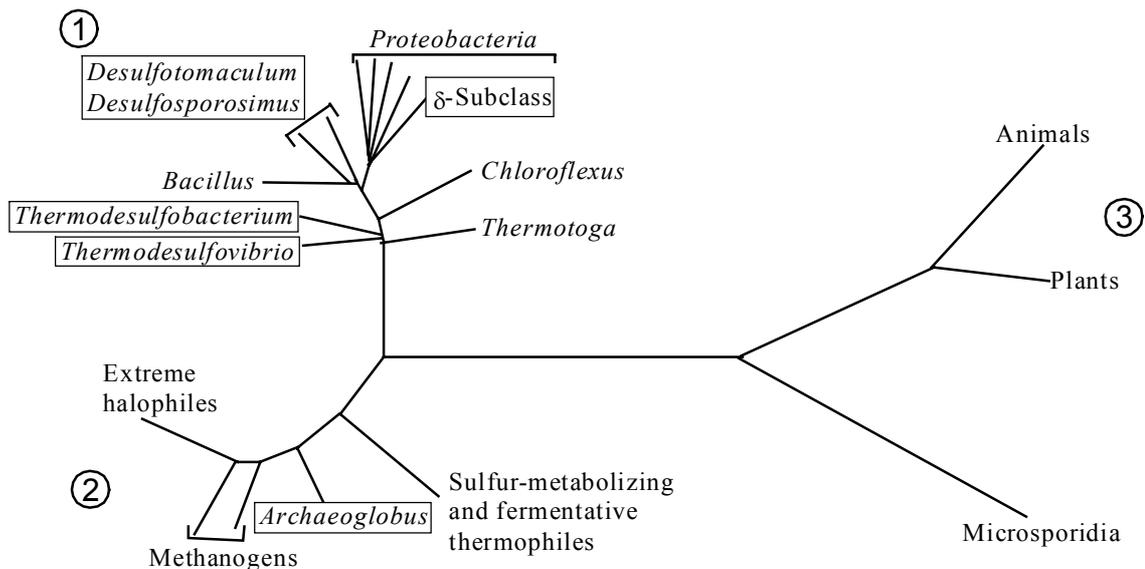


FIG. 2. Relationships of major groups of sulfate-reducing bacteria (framed) to other organisms, as based on 16S rRNA gene sequence analyses. (1) Bacteria; (2) Archaea; (3) Eukarya (adapted from Rabus et al. 2000).

Since sulfate is abundant in seawater (28 mM), sulfate reduction is more significant in the marine environment than in terrestrial aquatic habitats. Freshwater species are usually inhibited in medium with marine salinity (35 g NaCl/l) whereas marine species are often completely inhibited or die off in fresh water media (Widdel, 1988). Halophilic sulfate reducers such as *Desulfocella halophila* and *Desulfovibrio halophilus* isolated from

hypersaline environments grow optimally with 40–50 g NaCl/l and can tolerate up to 190 g NaCl/l (Caumette et al., 1991; Brandt et al., 1999).

Most SRB prefer a neutral environment and their growth is usually inhibited at pH values lower than 5–6 or higher than 9 (Widdel, 1988). Nevertheless, sulfate reduction has been observed in habitats with pH values between 3 and 4, as for instance in acid mine water (Tuttle et al., 1969; Tributsch et al., 1998). However, SRB isolated from such low-pH environments did not grow at pH values lower than 6. It was supposed that SRB in these acidic habitats grow in microniches with higher, physiologically more favorable pH values. Such microniches are probably maintained by the alkalization caused by the production of the proton-scavenging ions HS^- and HCO_3^- (Widdel, 1988). High sulfate reduction rates were also detected at pH 10 (Zavarzin et al., 1999). *Desulfonatronum lacustre* and *Desulfonatrovibrio hydrogenovorans* are representatives of SRB which exhibit optimal growth at high pH values (9.5 and above) (Zhilina et al., 1997; Pikuta et al., 1998).

Physiologically, SRB are separated into two main subgroups distinguished by their nutritional and biochemical characteristics. The incomplete oxidizers degrade organic substrates, such as lactate or higher fatty acids, to acetate as an end product. The complete oxidizers, in contrast, mineralize organic substrates including acetate to CO_2 (Postgate, 1984; Widdel and Bak, 1992; Rabus et al., 2000). Species of SRB commonly utilize low-molecular-mass compounds, including mono- and dicarboxylic acids, alcohols and aromatic compounds (Widdel and Pfennig, 1984; Widdel and Bak, 1992; Fauque et al., 1991; Hansen, 1994). They also oxidize saturated C_6 to C_{20} as well as aromatic hydrocarbons (Heider et al., 1999; Rabus et al., 2000; Spormann and Widdel, 2000). Many species of SRB, especially members of the genus *Desulfovibrio*, are able to utilize H_2 as electron donor for sulfate reduction (Widdel and Pfennig, 1984; Cypionka, 1995; Rabus et al., 2000). This capability has been supposed to play a key role in the hypothesized mechanism of anaerobic corrosion. The activating enzyme, hydrogenase, is therefore of special interest.

3.1.2 Hydrogenases in SRB

Sulfate reducers, particularly species of the genus *Desulfovibrio*, are able to utilize H_2 rapidly and with high affinity, even at an H_2 partial pressure down to approximately 0.02 Pa (Cord-Ruwisch et al., 1988). The enzyme hydrogenase (H_2 ase) catalyses the reversible reaction



The enzyme has been investigated biochemically as well as genetically mostly in *Desulfovibrio* species. SRB have been shown to possess species-specific combinations of three classes of hydrogenases which differ by their metal content and are accordingly designated as [Fe]-, [NiFe]- or [NiFeSe]-hydrogenases (Odom and Peck, 1984; Fauque et al., 1988; Rabus et al., 2000; Vignais et al. 2001). These three types of hydrogenases are remarkably different from each other in their catalytic activities, their molecular structures, and their sensitivity to specific inhibitors such as CO, NO, NO₂⁻ and acetylene (Fauque et al., 1988; Rabus et al., 2000). Screening of gene sequences suggested that [NiFe]-hydrogenase is more widespread among SRB than [Fe]- and [NiFeSe]-hydrogenases (Voordouw et al., 1990; Vignais et al. 2001). Most hydrogenases in SRB are located in the periplasmic space, and more than one type of hydrogenase is frequently observed (Glick et al., 1980; Odom and Peck, 1984; Fauque et al., 1988). It was postulated that sulfate reducers containing both [Fe]- and [NiFe]-hydrogenase have an important ecological advantage (Voordouw et al., 1993). The [NiFe]-hydrogenase has low H₂-uptake activity but high affinity to H₂ that allows *Desulfovibrio* spp. to survive in environments where H₂ occurs at low concentration. In contrast, [Fe]-hydrogenase has low affinity to H₂, however, its high H₂-uptake activity allows the bacteria to grow rapidly in environments with high H₂ concentration.

The oxidation of H₂ in *Desulfovibrio* is supposed to occur on the periplasmic side of the cytoplasmic membrane. Gained electrons are transported via electron carriers to the sulfate reduction machinery in the cytoplasm, thereby generating an electrochemical proton gradient for ATP synthesis (Fig. 3); further protons are presumably translocated during electron transport (Badziong and Thauer, 1980; Fitz and Cypionka, 1991; Cypionka, 1995; Rabus et al., 2000). The overall reaction can be written as:



The production of H₂, for instance in the absence of sulfate in methanogenic co-cultures, probably takes place in the cytoplasm (Odom and Peck, 1981; Lupton et al., 1984; Cypionka, 1995). In *Desulfovibrio vulgaris*, this function seems to be due to a cytoplasmic [NiFeSe]-hydrogenase (Rohde et al., 1990). However, [NiFeSe]-hydrogenase is not present in all H₂-producing SRB; H₂-production in such cases must, therefore, be catalyzed either by an unknown hydrogenase, or by the other known hydrogenases (Hatchikian et al., 1995). It has been speculated that certain *Desulfovibrio* species produce some H₂ in order to balance the redox state of the electron transport proteins (Lupton et al., 1984), or to scavenge H₂ released in the cytoplasm by periplasmic hydrogenases (hydrogen cycling) to generate a proton

gradient (Odom and Peck, 1981). Even though such a role of hydrogenases has been questioned (for overview see Widdel and Hansen, 1992). Some *Desulfovibrio* species apparently possess two different hydrogenases, a cytoplasmic and a periplasmic one. Other *Desulfovibrio* species, for example *Desulfovibrio vulgaris* (strain Groningen), however, possess only [NiFe]-hydrogenase and this single periplasmic enzyme seems to be responsible for both hydrogen uptake and production (Hatchikian et al., 1995).

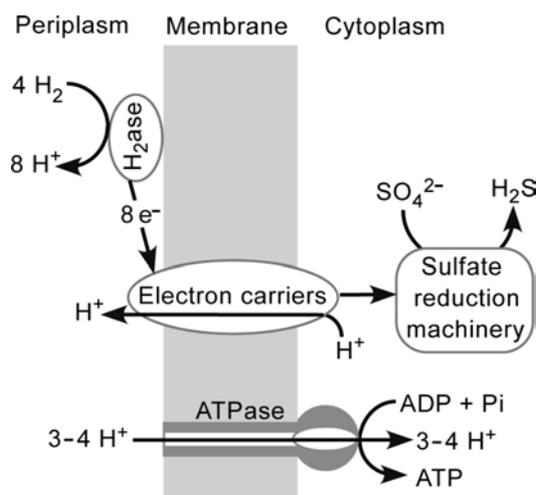


FIG. 3. Scheme of vectorial electron transport, proton translocation and ATP synthesis during sulfate reduction with H₂ in certain *Desulfovibrio* species (transport of sulfate is not included).

3.1.3 Mechanism of corrosion mediated by SRB

SRB are commonly detected at sites where anaerobic corrosion of iron occurs (Hamilton, 1985; Voordouw et al., 1990; Widdel, 1992a; Hamilton 1998a, 1998b). The corrosiveness of these organisms is partly due to their metabolic product H₂S (Costello, 1974; Widdel, 1992a; Lee et al. 1995), and partly due to a supposed more direct electrochemical effect termed cathodic depolarization (Von Wolzogen Kuehr and van der Vlugt, 1934).

Corrosion by H₂S. It was demonstrated that the rate of chemical corrosion was proportional to the concentration of H₂S added (Widdel, 1992a; Videla, 2000). H₂S accelerates iron corrosion by acting as a source of bound protons (eqn. 6) and by precipitation of Fe²⁺ as FeS (eqn. 7) (Costello, 1974; Lee et al., 1995):



The formed H_2 may be utilized further by SRB or by other H_2 -scavenging microorganisms.

Corrosion by cathodic depolarization. The more frequently discussed mechanism of corrosion mediated by SRB is a depolarization via oxidation of the cathodic hydrogen as formulated in the cathodic depolarization theory (Von Wolzogen Kuehr and van der Vlugt, 1934). In contact with water, metal becomes polarized by losing positive metal ions (anodic reaction). In the absence of oxygen, the liberated electrons reduce water-derived protons (cathodic reaction) to form hydrogen that remains on the metal surface, where a dynamic equilibrium is assumed to be established. Sulfate-reducing bacteria are supposed to remove the formed hydrogen (according to reaction 4), so that a net oxidation of the metal takes place. A scheme of this corrosion model is depicted in Fig. 4. However, it does not become clear in the various publications (for overview see references Miller, 1981; Widdel, 1992a) whether the depolarization is due to the consumption of atomic or molecular hydrogen. Often, the more undefined term *cathodic hydrogen* is used without further specification, or, as in Fig. 4, the symbol [H] is used to designate a hydrogen species of unknown structure.

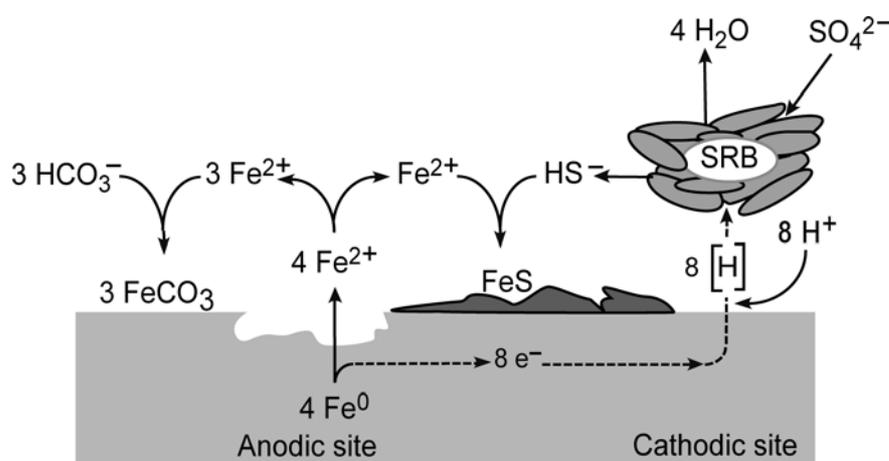


FIG. 4. Scheme of iron corrosion by SRB based on reactions as suggested by the cathodic depolarization theory. For convenience, the bacterial cells are drawn separately; in reality they live in intimate contact with the iron surface. At the cathodic site, reducing equivalents designated as [H] from the iron flow to the bacteria and are used for reduction of sulfate (SO_4^{2-}) to sulfide (H_2S). At the anodic site, only one fourth of the dissolved Fe^{2+} reacts stoichiometrically with H_2S to form FeS . In the presence of CO_2 and bicarbonate, as common in marine environments, the remaining Fe^{2+} precipitates as $FeCO_3$; in the absence of bicarbonate, the more soluble $Fe(OH)_2$ is formed.

The net reaction of corrosion is as following:



Experimental evidence to support the cathodic depolarization theory have been provided mostly with *Desulfovibrio* species since they utilize H₂ very effectively and can be easily cultivated under laboratory conditions (for a review see reference Pankhania, 1988). Iverson (1966) performed an experiment with cell suspensions of hydrogenase-positive *Desulfovibrio desulfuricans* and benzyl viologen as a substitute for sulfate. In the presence of iron, a reduction of benzyl viologen was observed, suggesting that hydrogenase was activated by the iron via cathodic hydrogen. Furthermore, in other experiments with benzyl viologen as alternative electron acceptor for the bacterial hydrogenase, Pankhania et al. (1986) found that the current density at a given electrode potential in the presence of *Desulfovibrio vulgaris* cells was always higher than in their absence. Booth and Tiller (1968) used electrochemical techniques to measure cathodic depolarization of steel with cell suspensions of different SRB. Their results clearly demonstrated that the hydrogenase-positive *Desulfovibrio vulgaris* could depolarize the cathode, whereas the hydrogenase-negative *Desulfotomaculum orientis* could not. Surprisingly, later it was found that *D. orientis* possesses hydrogenase in the intracellular rather than in the periplasmic space (Cypionka and Dilling, 1986); hence, the bacterium was capable to grow with H₂ and sulfate, but apparently did not accelerate corrosion. Hardy (1983) showed cathodic depolarization coupled to reduction of [³⁵S]-sulfate by using a resting cell suspension of H₂-grown SRB.

Cord-Ruwisch and Widdel (1986) also demonstrated oxidation of cathodic hydrogen with sulfate in different growing cultures of hydrogenase-positive *Desulfovibrio* species. These authors revealed that the process occurred only if an organic electron donor such as lactate was present. It was supposed that a simultaneous utilization of H₂ and the organic substrate took place. Also, the corrosive effect of sulfide from sulfate reduction with the organic substrate was considered. The rate of corrosion was found to be directly proportional to the metabolic activity of *Desulfovibrio* strains (assessed as hydrogenase and APS-reductase activity) as well as to their resistance to metal ions (Dzierzewicz et al., 1997). Interestingly, hydrogenases were found to be still active in old cultures of *Desulfovibrio vulgaris*, independent of the presence of viable cells (Chatelus et al., 1987). Under non-sulfide-producing conditions like during nitrate reduction, *Desulfovibrio* species could also oxidize cathodic hydrogen; however, the corrosion rate was usually much lower than during sulfate reduction (Rajagopal et al., 1988; Johnston et al., 1992), or was even not accelerated as in the case of *Desulfovibrio desulfuricans* (Feio et al., 2000). The hydrogenase-negative *Desulfobacter postgatei* did not exhibit a significant effect on corrosion (Gaylarde, 1992).

In corroding systems such as oil pipelines, SRB may be detected at high numbers (Cord-Ruwisch et al. 1987; Tardy-Jacquenod et al., 1996a; Magot et al., 2000). The corrosion rate was reported to depend largely on the total activity of hydrogenase within the biofilm rather on the bacterial population size (Bryant et al., 1991). The biofilm comprising SRB in a non-corroding pipeline had higher cell numbers but low hydrogenase activity and showed a low corrosion rate (0.48 mm iron oxidized per year). In contrast, biofilms with SRB in pipelines with intense corrosion (7.8 mm iron oxidized per year) had lower cell densities but much higher total hydrogenase activity. Often, hydrogenase genes have been subject to investigations with the goal to monitor corrosion under field conditions (Voordouw et al., 1990). However, the primers used for PCR amplification or the probes applied to detect hydrogenase genes *in situ* covered merely *Desulfovibrio* species (Voordouw et al., 1990; Wawer and Muyzwer, 1995). Therefore, such approaches cannot yield a complete picture of SRB associated with corroding iron *in situ*.

Further suggested mechanisms of corrosion by SRB. In an alternative model of anaerobic corrosion, it was proposed that the solid FeS formed on the metal surface becomes the cathodic site where hydrogen evolution from electrons and protons occurs more easily than on the metal (King and Miller, 1971; Miller, 1981; Widdel, 1992a). Already earlier, Booth et al. (1968) had studied cathodic depolarization of steel with *Desulfovibrio desulfuricans* growing on fumarate as electron acceptor in the presence or absence of chemically prepared FeS. The corrosion rate without added FeS was significantly lower than with FeS, thereby providing evidence for an involvement of FeS in cathodic depolarization.

Furthermore, sulfate reducers were supposed to accelerate corrosion via production of highly corrosive phosphorous compounds such as phosphine (H_3P) leading to the production of iron phosphide Fe_2P (Iverson, 1968; Iverson and Olson, 1984; Iverson, 2001). The precursor for the corrosive phosphorous compound has been speculated to be phosphate in yeast extract used for cultivation (Iverson, 1968) or inositol hexaphosphate, which is commonly found in plants, microorganisms and animal tissues (Iverson, 1998). It was demonstrated that *Desulfovibrio desulfuricans* growing with lactate increased the corrosion rates with increasing phosphate concentration in the medium (Weimer et al., 1988). However, in this study, vivianite ($Fe_3(PO_4)_2 \cdot 8 H_2O$) was identified as the main corrosion product, whereas iron phosphide (Fe_2P) was formed only in small quantities. There are also critical arguments against a formation of reduced phosphorous compounds. In comparison to sulfate reduction, the reduction of phosphate and other oxidized phosphorus species would require an

extremely strong reducing agent and thus much energy so that the bacteria could not benefit from such a reaction. It is, therefore, not likely that reduction of phosphorus species contributes to anaerobic corrosion (Widdel, 1992a). It must be kept in mind that technical iron regularly contains some iron phosphide which may hydrolyze to phosphine when iron gets in contact with an electrolyte (Widdel, 1992a; Glindemann et al., 1998; Roels and Verstraete, 2001).

Extracellular polymeric substances (EPS) produced by SRB have been shown to favour the attachment of cells to iron specimens and thereby accelerate corrosion (Beech and Cheung, 1995; Zinkevich et al., 1996; Beech et al., 1999; Fang et al., 2002). SRB with EPS of different composition were shown to cause different corrosion rates. In EPS released by a relatively aggressive *Desulfovibrio* strain, uronic acid was detected (Beech et al., 1994; 1998). Even EPS alone has been shown to corrode metal. A solution of 1% EPS produced by SRB enhanced corrosion up to 5 folds although cells of the SRB were not present (Chan et al., 2002). It has been claimed that EPS in corroding systems plays a role also as a trap of metal ions and therefore may stimulate the anodic reaction (Beech and Cheung, 1995; Beech and Tapper, 1999).

Also a direct utilization of electrons (liberated according to $\text{Fe} \rightarrow \text{Fe}^{2+} + 2 \text{e}^-$) by bacterial cells associated with the iron surface has been discussed as a possible mechanism of anaerobic corrosion (Widdel, 1992a). In such case, the involvement of electron carrying proteins localized in the outer membrane of cells would have to be postulated. Van Ommen Kloeke et al. (1995) discovered that *Desulfovibrio vulgaris* (Hildenborough) contained a high molecular mass cytochrome (HMC) in the outer membrane. Addition of this outer membrane fraction to medium containing metallic iron caused immediate acceleration in hydrogen production. It was assumed that electrons liberated from iron were directly transferred to HMC in the outer membrane without any intermediate and then donated to the periplasmic hydrogenases to form H_2 from protons. Evidence for a direct electron flow between metal and bacterial cells, but in the inverse direction, was obtained from iron respiring bacteria of the family *Geobacteraceae*, which like SRB belong to the δ -subclass of *Proteobacteria*. These organisms have been shown to conserve energy and grow by oxidizing organic compounds with a solid electrode serving as the electron acceptor (Bond et al., 2002; Bond and Lovley, 2003). Vice versa, one can envisage a direct transfer of electrons from the metal to the sulfate-reducing system in SRB. Such a direct withdrawal of electrons may be kinetically more favorable than consumption of the electrochemically formed H_2 ; however, experimental evidence for this assumption is required.

3.2 Corrosion by anaerobic microorganisms other than SRB

3.2.1 Corrosion by methanogenic archaea

Methanogenic archaea (methanogens) are obligate anaerobes which, like sulfate-reducing bacteria inhabit in oxygen-free environments. Most of the known species of methanogens live in moderate environments where they are nutritionally associated with fermentative and syntrophic H₂-producing microorganisms (Archer and Harris, 1986; Whitman et al., 1992). The species of methanogens described so far are separated into three major groups, according to their nutritional properties. These are (i) the hydrogenotrophic species that utilize H₂, formate, or certain alcohols for reduction of CO₂ to methane; (ii) the methylotrophic species that use C₁-compounds with methyl groups such as trimethylamine or methanol; and (iii) the acetoclastic methanogens that utilize mainly acetate for methane production (Bhatnagar et al., 1991; Whitman et al., 1992; Zinder, 1993; Garcia et al. 2000); however, these nutritional properties may overlap. The nutritional groups coincide to large extent with phylogenetic (16S rRNA based) lines of descent (Whitman et al., 1992; Garcia et al. 2000). Reduction of CO₂ to CH₄ with H₂ as electron donor is the energetically most favorable reaction (per mol of methane) under standard conditions (reaction 9) in comparison to the other reactions (Whitman et al., 1992; Deppenmeyer et al., 1996; Garcia et al., 2000).



It has been repeatedly demonstrated that sulfate reducers and methanogens in many natural environments compete with each other for H₂ and acetate. Since SRB usually exhibit higher maximum oxidation rates and affinity to H₂ and acetate in comparison to methanogens, sulfate reduction normally outcompetes methanogenesis in environments where degradable compounds and sulfate are available (Kristjanson et al., 1982; Jørgensen, 1983; Robinson and Tiedje, 1984; Lovley, 1985). Hence, methane formation is generally inhibited in marine sediments which contain high concentrations of sulfate (Jørgensen, 1983). In contrast, in freshwater sediments where sulfate concentration is low, methanogenesis is usually the dominant terminal process (Lovley et al., 1982; Lovley et al., 1987).

Also methanogens have been assumed to stimulate iron corrosion by consuming cathodic hydrogen, which results in the following net reaction:



Different strains of methanogens such as *Methanosarcina bakeri*, *Methanobacterium bryanti*, and *Methanospirillum hungatei* were shown to grow and produce methane in medium

containing iron powder or other metals as the only source of electrons (Daniels, 1987; Belay and Daniels, 1990). Growth of the methanogens with iron was largely dependent on the pH of the medium. Boothapy and Daniels (1991) showed that several strains of methanogens had pH optima for methane production between 6.2 and 7.0 if grown with H₂ and CO₂, but exhibited pH optima between 5.4 and 6.5 if iron served as the source of reducing equivalents. Apparently, the flux of protons being reduced to H₂ at low pH was higher than that under neutral or slightly alkaline conditions. Nevertheless, effective H₂-utilization did not always coincide with high corrosiveness. For instance, *Methanobacterium bryantii* utilized H₂ for methane formation more effectively but produced less methane than other species during growth with iron. In another study, some hydrogenotrophic methanogens like *Methanobacterium thermoautotrophicum* or *Methanosarcina frisia* could produce methane with iron but did not increase the concentration of Fe²⁺ formed from metallic iron in comparison to sterile controls (Dekena and Blotevogel, 1992). Generally, methanogenesis has been assumed to play a less important role in cathodic depolarization than sulfate reduction (Deckena and Blotevogel, 1990).

3.2.2 Corrosion by Fe(III)-reducing bacteria

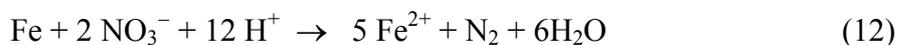
Dissimilatory Fe(III) reduction has been shown to compete successfully for H₂ not only with methanogenesis but also with sulfate reduction in natural habitats (Lovley and Phillips, 1987). Fe(III)-reducing bacteria have a higher affinity for H₂, and the change in free energy of Fe(III) reduction with H₂ is larger than that of sulfate reduction or methanogenesis (Lovley et al., 1994). It was proven that the Fe(III)-reducing bacterium *Shewanella putrefaciens*, which possesses hydrogenase, utilized H₂ for reduction of Fe(III) (in form of citrate salt) and simultaneously induced corrosion (Obuekwe et al. 1981b, 1981c; Dawood and Brözel, 1998). However, in many cases the depolarization was associated with the anode rather than with the cathode (Obuekwe et al., 1981c; Little et al., 1998). For instance, a strain of *Pseudomonas* isolated from corroding oil-pipeline reduced insoluble Fe(III) to soluble Fe(II) and in this way continuously exposed the metal surface to seawater and further corrosion (Obuekwe et al., 1981a). On the other hand, Fe(III)-reducing bacteria have been reported to reduce rather than to accelerate corrosion of steel (Potekhina et al., 1999). It was postulated that the bacteria continuously reduced Fe(III) to Fe(II) which acted as an oxygen scavenger and therefore inhibited corrosion (Dubiel et al., 2002).

3.2.3 Corrosion by nitrate-reducing bacteria

Nitrate reducing bacteria (denitrifiers) are also important members of the anaerobic microbial community. Reduction of nitrate to dinitrogen by denitrifiers is a multi-step process via nitrogen species of different redox states. Under oxygen-limited conditions, elemental iron served as an electron donor for chemical (abiotic) nitrate reduction to NH_4^+ (reaction 11) (Kielemoes et al. 2000). The principle of this reaction is known as Ulsch reduction which has formerly been applied for the chemical analysis of nitrate.



In a different, biological process, *Paracoccus denitrificans* used H_2 formed from the metal to reduce nitrate to N_2 , which resulted in the following net reaction (Till et al., 1998):



Similarly, *Escherichia coli*, which possesses hydrogenase, grew anaerobically with nitrate and H_2 and simultaneously accelerated corrosion (Mara and Williams, 1971). Nevertheless, nitrate reduction seems to be of minor importance in anaerobic metal corrosion in comparison to dissimilatory sulfate reduction (Kielemoes et al. 2000).

4. Goals of the present work

It is obvious from the given overview that sulfate-reducing bacteria are the most significant microorganisms in anaerobic corrosion of iron and steel, and two mechanisms are of prime importance. One mechanism is the chemical acceleration of corrosion by H_2S , the metabolic product of SRB. The other, more frequently discussed mechanism is the increased cathodic depolarization due to microbial scavenging of the reducing equivalents in a still unknown form.

Acceleration of corrosion by scavenging of hydrogen has been criticized by some authors (Costello, 1974; Hardy, 1983; Widdel, 1992a; Cord-Ruwisch et al., 1992, Cord-Ruwisch, 2000). It has been shown that H_2 formed on corroding iron at any partial pressure rapidly diffuses into the surrounding environment rather than remaining on the iron surface (Cord-Ruwisch et al., 1992). Hence, the removal of hydrogen from the iron surface is unlikely to be rate limiting for corrosion. Neither removal of H_2 nor the initial reduction of protons ($\text{e}^- + \text{H}^+ \rightarrow \text{H}$) is expected to delay corrosion (Miller, 1981; Widdel, 1992a; Cord-Ruwisch, 2000). Rather, the combination of atomic to molecular hydrogen ($2\text{H} \rightarrow \text{H}_2$) is the rate-

limiting step that causes the known overpotential of hydrogen formation on iron (Bockris and Reddy, 1977; Miller, 1981; Widdel, 1992a). SRB which directly stimulate corrosion must, therefore, accelerate the cathodic reaction by favoring the condensation of H atoms to H₂, or accept electrons from iron even directly without hydrogen as the intermediate (Widdel, 1992a; van Omen Kloeke et al., 1995). For performing such reactions the cells should live in very intimate contact with the iron surface. Furthermore, involvement of some redox components allowing electrons to flow through the outer cell barrier would have to be postulated (Widdel, 1992a).

During the past decades, the study of anaerobic corrosion of iron by SRB was largely based on experiments with *Desulfovibrio* species since they are widespread in nature and represent effective H₂ scavengers. Under field conditions, members of this group have been examined and identified as the apparently most important SRB involved in anaerobic corrosion (Voordouw et al., 1990; Telang et al., 1998). Nevertheless, clear evidence that *Desulfovibrio* species are the most important population and chief culprits in corroding systems has not been provided.

If scavenge of reducing equivalents (as hydrogen or via other carriers) is a decisive process in anaerobic corrosion, also methanogens as the natural counterparts of SRB should in principle be able to stimulate corrosion purely via a cathodic depolarization reaction. Although methane production with metallic iron has been demonstrated with different strains of methanogens, acceleration of corrosion due to this process was not really evident (Deckena and Blotevogel, 1992; Jones and Amy, 2000).

Obviously, there is presently no mechanistic model of anaerobic corrosion that is in agreement with all the reported observations and arguments. The goal of my work was, therefore, to elucidate some mechanisms and principles in the process of anaerobic biocorrosion in more detail, especially the involved key organisms and the form of reducing equivalents that are transferred from the iron surface to the microbial cells. To reach this goal my approaches were as following:

- It was investigated whether certain specific, highly corrosive groups of sulfate-reducing and methanogenic microorganisms developed in anoxic enrichment cultures directly with metallic iron (Fe) as the only source of electrons for sulfate reduction or methanogenesis, respectively. For control, conventional enrichment cultures with H₂ (without iron) were carried out in parallel.
- Furthermore, the isolation and physiological characterization of strains from the enrichment cultures were of importance. Corrosion experiments with obtained pure

cultures were expected to confirm whether or not these cultures can be regarded as main culprits in the corrosion process in the enrichment cultures with iron and possibly also *in situ*.

- Molecular techniques based on 16S rRNA analysis and fluorescence *in situ* hybridization were applied to reveal the phylogeny of the new isolates and their abundance in the original enrichment cultures with metallic iron.

B Results of the present study

1. Anaerobic corrosion by sulfate-reducing bacteria

In laboratory corrosion experiments, *Desulfovibrio* species usually serve as model organisms (Booth and Tiller, 1968; Hardy, 1983; Pankhania et al., 1986; Cord-Ruwisch and Widdel, 1986; Deckena and Blotevogel, 1990; Beech et al., 1994). Accordingly, also monitoring techniques under field conditions often target species of the genus *Desulfovibrio* which are regarded as the main cause of anaerobic corrosion (Miller, 1981; Voordouw et al., 1990; Telang et al., 1998). In fact, *Desulfovibrio* species have been revealed as the apparently most abundant population in corrosive biofilms (Zhang and Fang, 2001), and several sulfate reducers isolated from corrosive environments have been identified as *Desulfovibrio* species (Tardy-Jaquenod et al., 1996b; Feio et al., 1998; Magot, 2000). Nevertheless, it has not been proven that *Desulfovibrio* is the most important type of SRB involved in the corrosion of iron, and little is known about a possible role of SRB other than *Desulfovibrio* in anaerobic corrosion. The present study was performed to clarify whether only *Desulfovibrio* species or also, or even preferentially, other sulfate-reducing bacteria develop on iron under sulfate-reducing condition. Once the key organisms have been identified and isolated, studies on their physiological properties could help to elucidate detailed mechanisms in the still insufficiently understood process of anaerobic corrosion.

1.1 Enrichment of SRB with metallic iron (Fe) as the only source of electrons

The present study was focused on corrosion in marine environments where the process is usually most intensive due to high concentration of sulfate and high activities of SRB. In the literature, corrosion experiments with SRB commonly employed medium containing organic substrates such as lactate to support growth of the bacteria in the presence of metallic iron (Hardy, 1983; Pankhania et al., 1986; Cord-Ruwisch and Widdel, 1986). However, in such experiments it is difficult to clarify whether the accelerated corrosion is due to direct interaction between the SRB with the iron or to chemical corrosion by H₂S produced by the bacteria with the organic substrates. To circumvent this problem, experiments in the present study were performed with metallic iron as the only source of electrons for sulfate reduction without any organic substrate.

The enrichments of SRB were carried out in glass bottles containing anoxic seawater medium with iron as the only source of electrons. $\text{CO}_2/\text{HCO}_3^-$ alone or $\text{CO}_2/\text{HCO}_3^-$ with acetate added at low concentration (1 mM) serving as carbon source. Sulfide-rich marine sediment samples from geographically distant sites (North Sea, Germany and Halong Bay, Vietnam) were used as initial source of SRB. For control, parallel enrichment cultures were carried out with H_2 as electron donor instead of iron. To enrich SRB in these controls at sulfate reduction rates comparable to those with iron, H_2 was slowly provided via diffusion through a silicon membrane (part II, 2, Fig. 1).

After several successive transfers, sediment free enrichment cultures were obtained. Sulfate was reduced at similar rates in the enrichment cultures with iron or with H_2 (Fig. 5). Most intensive sulfate reduction occurred within the first two weeks of incubation. A pronounced precipitation of FeS was observed in the culture bottles of the enrichment cultures with iron, especially when CO_2 alone served as carbon source (part II, 2, Fig. 2). Whereas cells in the enrichment culture with H_2 grew densely in the liquid medium, cells in the enrichment cultures with iron were detected only occasionally in free medium. Obviously, in the presence of iron, most of cells tended to associate with the iron surface.

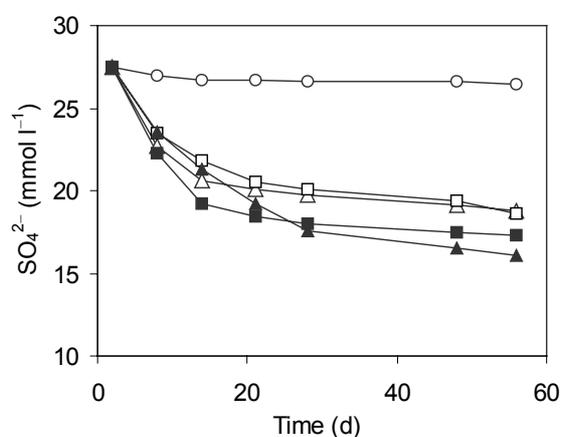


FIG. 5. Sulfate reduction in the enrichment cultures with sediment from North Sea. Sulfate reduction rates were comparable in the enrichment cultures with iron (\square) and with slowly provided H_2 (Δ). No sulfate was reduced in the sterile control (\circ). In enrichment cultures containing in addition acetate as organic carbon source (with iron [\blacksquare] or with H_2 [\blacktriangle]), sulfate reduction rate was slightly higher.

1.2 Molecular analysis of bacterial communities in the enrichment cultures

Total DNA extracted from the enrichment cultures of SRB was used to analyze the bacterial communities via denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S

rRNA gene fragments. The universal primer set GM5F (with GC clamp) and 907R, which is specific for the domain *Bacteria*, was used to obtain 550 bp fragments of the 16S rRNA gene (Muyzer et al., 1993; 1995). Significant differences in the DGGE profiles were observed between the enrichment cultures with iron and those with H₂ (part II, 2, Figs. 3, 4). Analysis of the DGGE band sequences showed that the enrichment cultures with H₂ (with or without acetate) yielded mainly *Desulfovibrio*-related SRB. In contrast, all the enrichment cultures with iron harboured a *Desulfobacterium*-related population besides *Desulfovibrio*-related species, which seemed to be minor. Furthermore, the *Desulfobacterium*-related population was detected in iron-grown enrichment cultures with marine sediment samples from North Sea (Germany) as well as from Halong Bay (Vietnam), indicating that this type of bacterium might be widely distributed. In conclusion, a specific population of SRB which related to *Desulfobacterium* species was apparently enriched with iron as the only electron donor for sulfate reduction.

The process of corrosion leads to a strong increase of the pH due to the stoichiometrically significant consumption of protons (reaction 8). It might, therefore, be suspected that *Desulfobacterium*-related population developed exclusively in the iron-grown enrichment cultures due to the alkaline conditions rather than due to the utilization of reducing equivalents from iron. To prove this possibility, the enrichment cultures with iron were subsequently grown with H₂ as electron donor instead of iron in alkaline medium (pH 8.5). In this experiment, the bicarbonate buffered salt medium (Widdel and Bak, 1992) was used instead of conventional seawater medium to minimize precipitation of alkaline earth minerals at high pH values. FeS and FeCO₃ were also added according to the amounts of ferrous iron that could be released from the iron due the attack of the enrichment culture (calculated from sulfate reduction). However, microscopic examination upon growth revealed development of a cell type in these enrichment cultures that differed from those selected with iron. DGGE analysis showed that the enrichment cultures with H₂ under alkaline conditions yielded a new band affiliating to *Desulfocapsa* species, whereas the band represented for the *Desulfobacterium*-related population disappeared gradually.

1.3 Isolation and characterization of SRB from the enrichment cultures

For the isolation of SRB from the enrichment cultures with iron, precipitate flocks on the iron surface were collected, homogenized (anaerobically) and used as starting inoculum in liquid serial dilution with mineral medium and iron granules. The bacterial cultures from the tube of

the highest dilution that showed growth were purified further via agar dilution series with a mixture of lactate, propionate, butyrate, pyruvate, ethanol and H₂.

TABLE 1. Physiological and phylogenetic characterization of the new isolates of SRB

Characteristics	Strain IS4	Strain IS5	Strain HS2
Enriched / isolated with	Iron (+ CO ₂)	Iron (+ CO ₂ + acetate)	Hydrogen (+ CO ₂ + acetate)
Phylogenetic affiliation (16S rRNA gene based)	<i>Desulfobacterium catecholicum</i> (95%)	<i>Desulfovibrio senezii</i> (92%)	<i>Desulfovibrio caledoniensis</i> (98%)
G + C content (mol%)	51.9	55.8	ND
Morphology	Rods	Vibrioids	Vibrioids
Size (µm)	1 × 4–8	0.5–1 × 4–8	0.5 × 2–4
Optimum temperature (°C)	28–30	28–30	28–30
Optimum pH	8.9–9.1	7.8–8.2	7.2–7.5
Optimum salinity (g NaCl/l)	10–15	10–15	ND
Electron donors utilized			
Fe	+	+	–
H ₂ (+ CO ₂)	+	–	–
H ₂ (+ CO ₂ + acetate)	+	+	+
Formate	+	+	ND
Lactate	+	+	+
Pyruvate	+	+	+
Electron acceptors tested			
Sulfate	+	+	+
Sulfite	–	+	ND
Thiosulfate	–	+	ND

Symbols: +, utilized; –, not utilized; ND, not determined.

Colonies of curved, *Desulfovibrio*-like cells appeared first, followed by colonies of rod-shaped cells. Strain IS4 and IS5 were representatives for these rod-shaped and curved cells, respectively, isolated from iron-grown enrichment cultures with sediment from North Sea. In addition, a rod-shaped sulfate-reducing bacterium, strain HL-IS1 was isolated from enrichment culture with iron and Halong Bay sediment via the same procedure. Isolation of

SRB from the enrichment cultures with H₂ was carried out directly via repeated agar dilution series with H₂ as electron donor and CO₂ with acetate added as carbon source. Only one type of colony with small curved, *Desulfovibrio*-like cells developed, yielding strain HS2 as a representative isolate. Analysis of 16S RNA gene sequences of the isolates revealed that the rod-shaped isolates IS4 and HL-IS1 represented different strains of the same species which exhibited relationship to the genus *Desulfobacterium*, the closest cultivated relative being *Desulfobacterium catecholicum* (95% sequence similarity) (Part II, 1, Fig. 2). Strain IS4 was chosen as type strain for further studies. Strains IS5 and HS2 were apparently *Desulfovibrio* species. Based on the phylogenetic status and physiological properties, strain IS4 is regarded as a new species of the genus *Desulfobacterium*; the name *Desulfobacterium corrodens* is proposed. Strain IS5 exhibited significant phylogenetic distance to its closest cultivated relative *Desulfovibrio senezii* (92% similarity in sequence of 16S rRNA gene) and is, therefore, regarded as a new species of the genus *Desulfovibrio*; the name *Desulfovibrio ferrophilus* is proposed. Physiological and phylogenetic characteristics of the newly isolated sulfate reducers are shown in table 1.

A control experiment was performed to check whether selection of SRB in the enrichment cultures with iron was due to the presence of iron or to resistance against FeCO₃, a main corrosion product that may be slightly toxic. In this experiment, the newly isolated SRB and *Desulfovibrio salexigens* were grown in lactate-sulfate medium containing different amounts of FeCO₃. The growth rate indeed decreased with increasing amounts of FeCO₃, but this effect was observed evenly with all the tested strains, i.e. with the newly isolated SRB and *Desulfovibrio* species from strain collection. Selection of SRB as represented by strain IS4 due to selective inhibition of *Desulfovibrio* species and other competing SRB by FeCO₃ can be, therefore, excluded.

1.4 *In situ* identification of SRB in the enrichment cultures with metallic iron

The bacterial communities associated with iron surface in the enrichment culture with iron and CO₂ was analyzed by fluorescence *in situ* hybridization (FISH). Since direct hybridization on the iron surface was impossible, precipitated flocks of FeS were collected from the corroding surface, fixed in formaldehyde, and filtrated onto polycarbonate filters (Millipore; pore size, 0.2 µm), which were used for hybridization. An oligonucleotide probe (Dbm212) targeting specifically rRNA of strain IS4 was designed. This probe does not hybridize with rRNA from *Desulfobacterium catecholicum*, the phylogenetically closest relative of strain

IS4. To get strong signals of the hybridized cells and to avoid interference by the background fluorescence, a probe-linked to horseradish peroxidase (HRP) and the newly described hybridization technique CARD-FISH (Pernthaler et al., 2002) were applied. More than 90% of the DAPI stained cells in the sessile community exhibited the same morphology as strain IS4 and hybridized with probe Dbm212 (part II, 1, Fig. 1). Furthermore, hybridization with cy3-labeled probe DSV698 (Manz et al., 2000) was carried out with the same samples to identify *Desulfovibrio* species. Curved, *Desulfovibrio*-like cells were detected occasionally by DAPI staining, however no hybridization signal was observed. The *Desulfobacterium*-related sulfate reducers represented by strain IS4 apparently predominated in the enrichment culture with iron, whereas *Desulfovibrio* species were of minority. This result was in contrast to previous views which suggest crucial role of *Desulfovibrio* species in anaerobic corrosion (Miller, 1981; Cord-Ruwisch et al., 1987; Pankhania, 1988). The new type of SRB represented by strain IS4 was, therefore, supposed to play a promoting role in the process of anaerobic corrosion under the employed conditions.

1.5 Study of corrosion by new isolates of SRB

1.5.1 Capability of sulfate reduction with metallic iron

Growth and sulfate reduction with iron were compared among the new isolates of SRB to reveal possible differences in their ability to gain reducing equivalents from the iron surface and consequently to stimulate corrosion. Growth experiments were carried out in bottles containing anoxic mineral medium and iron granules as the only source of reducing equivalents. Acetate (1 mM) was added as a carbon source. For comparison, we included hydrogenase-positive marine sulfate-reducing bacteria *Desulfobacterium catecholicum* and *Desulfovibrio salexigens* and the freshwater strain *Desulfovibrio vulgaris* (strain Hildenborough) which is frequently used in corrosion experiments.

Strain IS4 reduced sulfate with iron more rapidly than the *Desulfovibrio* strains IS5 and HS2, as well as *Desulfovibrio salexigens*, *Desulfobacterium catecholicum* and *Desulfovibrio vulgaris* (part II, 1, Fig.3). Next to strain IS4, the *Desulfovibrio* strain IS5 reduced sulfate with iron most rapidly. The rate of sulfate reduction by strain IS4 with iron was very close to that by the enrichment culture from which the strain had been isolated. One may, therefore, assume that this strain represents the dominating type of sulfate-reducing bacterium in the enrichment culture and is mainly responsible for the oxidation of iron therein. During growth with iron, strain IS4 produced remarkable layers of dark FeS sticking to the glass wall in the

culture bottles, whereas FeS produced by the *Desulfovibrio* strains apparently covered only the iron granules (part II, 2, Fig. 2).

Furthermore, growth of strain IS4 seemed to be largely dependent on the availability of fresh iron surface. Adding fresh iron granules stimulated sulfate reduction by this strain with iron, which significantly decreased with incubation time (Fig. 6).

On the other hand, with substrates commonly used by SRB such as lactate or H₂, strain IS4 grew significantly slower than the *Desulfovibrio* strains IS5 and HS2, or the type strain *Desulfovibrio salexigens* (part II, 2, Fig.5). This result could give an explanation for the selective enrichment of *Desulfovibrio*- or *Desulfovibrio* related species in iron- or H₂-grown enrichment cultures, respectively. The *Desulfovibrio*-related SRB represented by strain IS4 grew faster with iron therefore became dominating in enrichment culture with iron, whereas in the enrichment cultures with H₂ they were readily outcompeted by effectively H₂-scavenging *Desulfovibrio* species. One may conclude that strain IS4 is well adapted to growth with iron and is, therefore, a particularly “corrosive” sulfate-reducing bacterium.

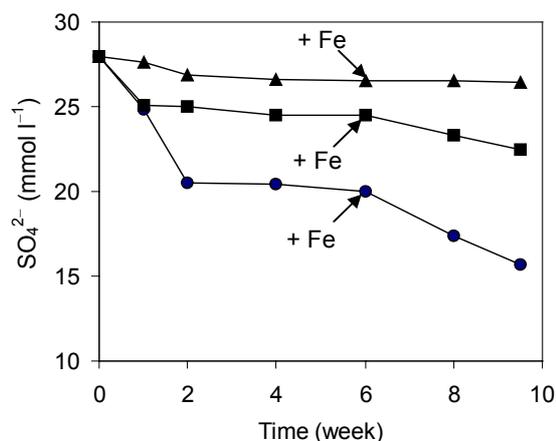


FIG. 6. Effect of the availability of a fresh iron surface on growth of new isolates. The rate of sulfate reduction by strain IS4 (●) decreased significantly after two weeks of incubation and increased again if new iron granules were added. This effect was less significant for *Desulfovibrio* strain IS5 (■) and was not observed at all with *Desulfovibrio* strain HS2 (▲).

During growth of the SRB with iron as the source of electrons, the pH value of the medium always increased due to significant consumption of protons (see equation 8). The highest pH value of the medium at the end of growth was detected in the culture of strain IS4 grown with iron. This strain reached pH values above 9 whereas growth of the other strains ceased at pH 8–8.5. In this context also the optimal pH values for growth of the new isolates

were determined. For this purpose, a salt-water medium with a lower magnesium and calcium concentration than seawater medium was used to avoid precipitation. Lactate was used as electron donor and carbon source. Strain IS4 grew best at pH around 9, whereas all the tested *Desulfovibrio* strains exhibited lower pH optima. This special characteristic could be an advantage of strain IS4 for its growth on corroding iron, particularly in the microniches of pits, where access of less alkaline water may be limited.

1.5.2 Rate of sulfate reduction with metallic iron

Rates of sulfate reduction with iron by different strains of SRB were compared to reveal possible stimulating effects of bacterial cells on depolarization. Stoichiometrically, four mol H₂ are needed to reduce one mol sulfate (reaction 5). The rate of sulfate reduction by *Desulfovibrio salexigens*, *Desulfovibrio vulgaris* or *Desulfovibrio* isolate HS2 could be accounted for by the rate of chemical H₂ formation; hence sulfate reduction by these species was a subsequent reaction following H₂ formation with iron. In contrast, strain IS4 growing with iron exhibited a sulfate reduction rate significantly exceeding that expected with the chemically formed H₂ (Fig. 7).

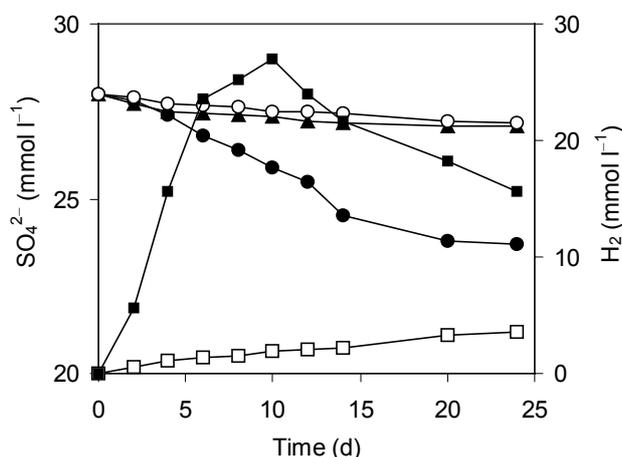


FIG. 7. Sulfate reduction and stimulated H₂ formation by strain IS4 during growth with iron. The strain reduced sulfate (●) at a much higher rate than that could be achieved with the chemically formed H₂ (○) serving as the only electron donor. Sulfate reduction by *Desulfovibrio salexigens* (▲) was comparable to that expected with the rate of chemical H₂ formation. Moreover, even a stimulated formation of H₂ (◆) was observed in comparison to the sterile control (◇) (see also 1.5.3).

Apparently, strain IS4 must have not merely used the chemically formed H₂ for the sulfate reduction but gained reducing equivalents from the iron in a more direct way, without hydrogen as an intermediate.

1.5.3 Hydrogenase activity and accelerated H₂ formation with metallic iron

Considering that the enzyme hydrogenase has been repeatedly assumed to play an important role in anaerobic corrosion (Iverson, 1966; Chateleus et al., 1987; Bryant et al., 1991, Dzierzewicz et al., 1997), specific activities of hydrogenases were determined in the cultures of the new isolates of SRB (Tab. 2). Interestingly, strain IS4 possessed less hydrogenase activity but reduced sulfate with iron more rapidly than the *Desulfovibrio* strains IS5 and HS2. In this case, the specific activity of hydrogenase did not positively correlate with the corrosiveness of the SRB as reported elsewhere (Bryant et al., 1991; Dzierzewicz et al., 1997).

TABLE 2. Hydrogenase activity in the new isolates of SRB. Since cells could not be harvested from media with iron-granules, cultures for this experiment were grown on lactate or H₂.

Strain	Hydrogenase activity ($\mu\text{mol min}^{-1}\text{mg}^{-1}$)
IS4	12.3
IS5	27.9
HS2	28.2

A surprising observation during growth of strain IS4 with iron was that gas bubbles were formed at the iron surface. Such gas bubbles were usually observed only in the sterile controls containing iron and medium, but not in the presence of H₂-utilizing SRB. Chemical analysis revealed that H₂ was formed and accumulated during early growth phase in the culture bottle of strain IS4 growing with iron. The rate of H₂ formation with iron in the presence of this strain was even significantly higher than in the sterile control (Fig. 7). Such formation of H₂ was neither observed in cultures of *Desulfovibrio* isolates IS5 and HS2, nor in the culture of type strain *Desulfovibrio salexigens* if incubated with iron.

Further experiments were carried out to measure to which extent H₂S or FeS alone can stimulate the chemical formation of H₂ with iron in aqueous medium; such stimulation has been reported by King and Miller (1971), Costello (1974) and Widdel (1992a). In controls

without bacteria, H₂S was added at a concentration comparable to that produced by strain IS4 during growth with iron. In this experiment, FeS was formed according to the reaction: Fe + H₂S → FeS + H₂. The added H₂S indeed stimulated the production of H₂ from iron (Fig. 8). After a while, probably when H₂S had been depleted, the stimulation of H₂ formation decreased. Alternatively, FeS was also added from a separately prepared slurry (from FeSO₄ and Na₂S solutions). Similarly, the FeS precipitated on the iron surface slightly accelerated cathodic depolarization, i.e. the formation of H₂ (H⁺ reduction to H₂). However, chemical H₂ production in the presence of sulfide species (as H₂S or FeS added) was always slower than the reaction achieved by the newly isolated strain IS4.

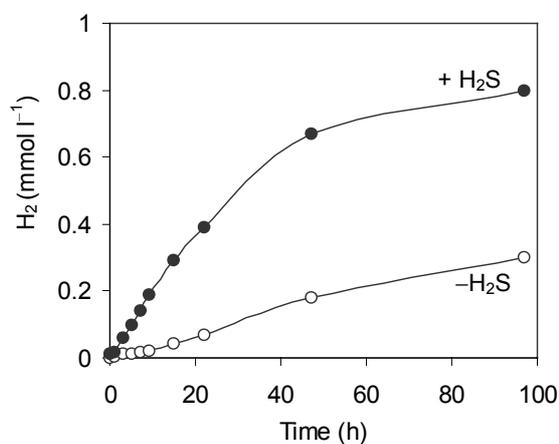


FIG. 8. Acceleration of H₂ formation from metallic iron by H₂S. A 250 ml glass bottle with 150 ml anoxic medium contained 30 g iron granules and closed with butyl-rubber stopper under N₂/CO₂ (90/10 vol/vol) atmosphere. At time zero, 1 ml of a 1 M solution of Na₂S with 1.5 ml of a 1 M solution of HCl was injected (yielded a neutral pH of 7–7.2). The amount of H₂ is attributed to the aqueous phase.

The accelerated H₂ formation with iron was observed not only in pure culture of strain IS4 but also in the enrichment culture. However, the H₂ produced in this enrichment culture was rapidly consumed after a while, apparently due to the subsequent development of effectively H₂-utilizing microorganisms such as *Desulfovibrio* species. In a defined mixed culture of strains IS4 and IS5 grown with iron, no H₂ accumulation was observed, whereas the sulfate reduction rate was slightly higher than in the culture of strain IS4 alone. In the presence of *Desulfovibrio* strain HS2 or *Desulfovibrio salexigens* the H₂ formed with iron was probably immediately utilized by the cells, and, therefore, could not be detected.

The finding that strain IS4 accelerated H₂ formation with iron may give an explanation for the exceeding FeS precipitation in the culture bottle of this strain or the enrichment culture during growth with iron. Surface-attached cells of this bacterium reduce sulfate to sulfide that is immediately precipitated as FeS on the iron. Simultaneously, the attached cells also release excess reducing equivalents as H₂ which is subsequently used by other cells of strain IS4 or *Desulfovibrio* (in the enrichment culture) for the reduction of sulfate to form additional ferrous sulfide throughout the bottle. Furthermore, the transient accumulation of H₂ during growth of strain IS4 with iron showed that H₂ produced from iron surface cannot be the rate-limiting step in the process of corrosion as suggested in the cathodic depolarization theory.

The accelerated production of H₂ with iron is probably catalyzed by the bacterial hydrogenase operating in the reverse direction. Although hydrogenases are present in different groups of SRB, this enzyme has been studied intensively only in *Desulfovibrio* species. Most of the knowledge about gene sequences, catalytic properties and enzyme structure stems from investigations of *Desulfovibrio* species (Van Dongen, 1995; Vignais, 2001). In the present study, primer sets specific for [NiFe]-hydrogenase (Wawer and Muyzer, 1995) and for [Fe]-hydrogenase designed on the basis of the available hydrogenase gene sequences from *Desulfovibrio* were used; however, the PCR was not successful. Also, Southern blot of EcoRI-digested DNA from strain IS4 with DNA probes specifically designed for [NiFe]- or [Fe]-hydrogenase did not yield hybridization signal, again indicating that there are remarkable differences in the genetic structure of hydrogenases in strain IS4 and other sulfate reducers. More detailed investigations into the primary structure of hydrogenases in strain IS4 were beyond the frame of the present work.

1.5.4 Analyses of the corroding iron surface

Iron metal in contact with the enrichment cultures of SRB showed significant changes on the surface due to corrosion. The enrichment culture with CO₂ and acetate as carbon sources apparently affected the iron surface to a less extent (Fig. 9A) than the enrichment culture with CO₂ alone (Fig. 9B). There is presently no explanation for the apparently higher corrosiveness of the autotrophic culture.

If well-polished iron coupons were incubated with pure cultures of strain IS4 or IS5, thick layers of precipitates with embedded cells could be detected by scanning electron microscopy (SEM) (part II, 1, Fig. 1). In these layers, cells of strain IS4 were present at higher abundance than cells of strain IS5. This observation again indicated that growth of strain IS4

occurred mostly in association with the iron surface. Additionally, X-ray photoelectron spectroscopy (XPS) of the corrosion products in the layer formed by strain IS4 revealed that the iron species were mainly FeS and FeCO₃.

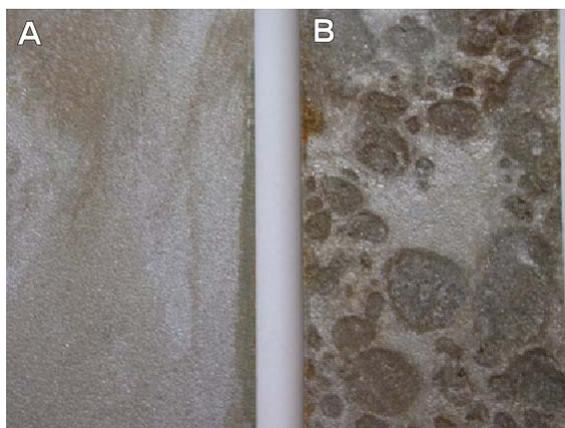


FIG. 9. Iron coupons immersed in the enrichment culture with (A) acetate and CO₂ as carbon sources and (B) with CO₂ alone as carbon source after 10 months of incubation. To remove corrosion products from the iron surface, the iron coupons were dipped shortly in 2 M HCl solution containing hexamin (10%, wt/vol), repeatedly rinsed in anoxic water, dried and stored under an N₂ atmosphere.

2. Anaerobic corrosion by methanogens

The possible role of methanogens in anaerobic iron corrosion has been studied to a lesser extent than the role of SRB in this process. According to the cathodic depolarization theory, also methanogens should, in principle, be able to accelerate corrosion if they can make use of cathodic hydrogen. Corrosion experiments with different pure cultures of methanogens, however, showed contradictory results (Daniels et al., 1987; Belay and Daniels, 1990; Deckena and Blotevogel, 1990; Deckena and Blotevogel, 1992; Boopathy and Daniels, 1991). In the present study, the possible role of methanogens in anaerobic corrosion was investigated in a similar manner as with SRB, i. e. by using enrichment cultures on metallic iron (Fe) as the starting material.

2.1 Enrichment of methanogens with metallic iron as the only source of electrons

Enrichment of methanogens was performed in anoxic seawater medium (Widdel and Back, 1992) without sulfate and with iron as the only source of electrons. A sediment sample from the North Sea was used as the initial inoculum.

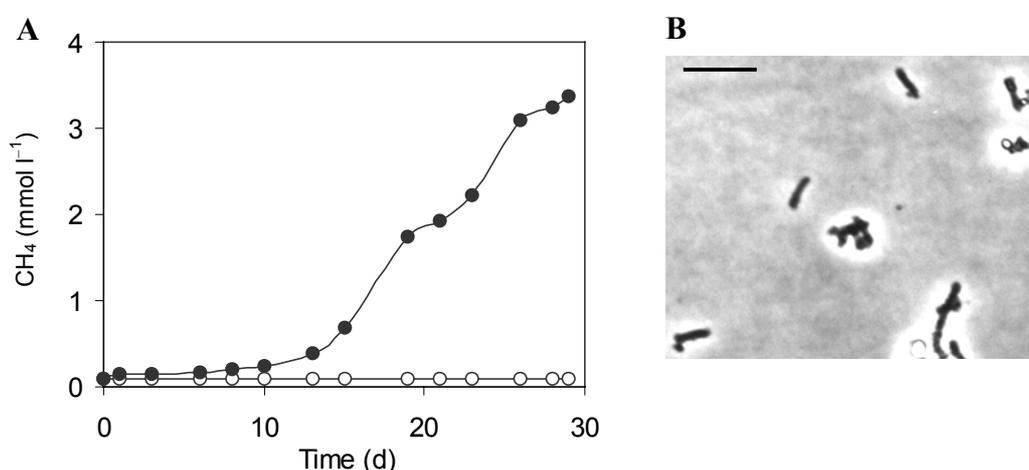


FIG. 10. (A) Methane production in the sediment-free enrichment culture with metallic iron. The experiment was carried out in glass bottle (250 ml) containing 150 ml seawater medium (without sulfate). Iron granules (diameter approximately 2 mm) were added (30 g) as the source of electrons. A high amount of methane was produced by the enrichment culture (●) in comparison to the sterile control (○). For convenience, the indicated methane is attributed exclusively to the aqueous phase. (B) Phase-contrast photomicrograph of cells from the methanogenic enrichment culture with iron. Bar, 10 μm.

Methane production with iron occurred slowly such that successful transfer of the enrichment culture was only possible at time intervals of 4 to 6 months. In this enrichment culture, pronounced methane production (Fig. 10A) concomitantly with Fe²⁺ precipitation as white FeCO₃ was observed. Microscopic observation revealed mainly rod-shaped cells (Fig. 10B) that showed autofluorescence indicative of coenzyme F₄₂₀.

2.2 Molecular analyses of microbial community in the enrichment culture

Two primer sets, ARC516F (Knittel, unpublished)/ARC958R (DeLong, 1992) and ARC344F/ARC915R (Casamayor et al., 2000), which are specific for the domain *Archaea*, were used to obtain PCR products from 16S rRNA genes of 442 and 571 bp, respectively, from the DNA pool of the methanogenic enrichment culture. For some unknown reasons, DGGE analysis was not successful with the obtained PCR products. Therefore, another approach was attempted, in which distinct PCR products were separated in conventional agarose gel containing H.A.-Yellow (bizbenzimidazole-polyethylene glycol) which binds preferentially to A and T sequence motifs in the DNA (Wawer et al., 1995; Müller et al., 1997). In this experiment, longer PCR fragments (approximately 1000 bp) were obtained with the primer pair ARC344F (Casamayor et al., 2000)/ARC1406R (Huber et al., 2002). In the agarose gel with H.A.-Yellow a single band was detected (Fig. 11) the sequence of which showed significant similarity to sequences of *Methanobacterium* and *Methanobrevibacter* (96% sequence similarity to each genus).

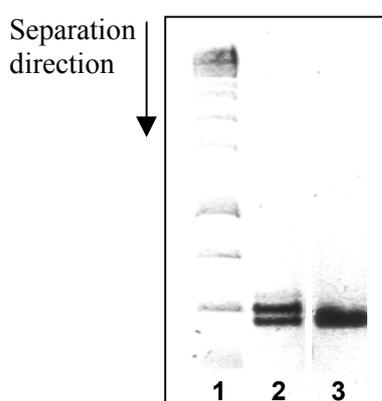


FIG. 11. Analysis of PCR-amplified 16S rRNA gene fragments in an agarose gel containing H.A.-Yellow. Lane 1, DNA marker; lane 2, defined mixed culture of three methanogenic species (*Methanosarcina mazei*, *Methanospirillum hungatei* and *Methanocarcina acetivorans*); lane 3, methanogenic enrichment culture with iron. The band yielded in the enrichment culture was excised and sequenced.

2.3 Isolation and characterization of a methanogenic pure culture from the enrichment culture

The methanogenic enrichment culture with iron was subjected to serial dilution in liquid medium with iron granules. CO₂ served as the only source of carbon and terminal electron acceptor. Significant methane formation was detected at dilutions as high as 10⁻⁷. Microscopic observation revealed only one morphological cell type in the culture at a dilution of 10⁻⁷. PCR using the primer pair ARC344F and ARC1406R yielded a single fragment of the 16S rRNA gene (approximately 1000 bp). Sequencing showed high similarity (96 %) to sequences of the genera *Methanobacterium* and *Methanobrevibacter*. The methanogenic culture obtained at the dilution of 10⁻⁷ was assumed to be a new type of methanogenic archaeon and was designated as strain IM1. So far, however, it was impossible to obtain a full-length 16S rRNA gene sequence of strain IM1 by using the available archaeal primer sets (Tab. 3). One may conclude that this culture represents a hitherto unknown line of descent within the methanogenic Archaea.

TABLE 3. Primers used for PCR to obtain 16S rRNA gene fragments from the new methanogenic strain IM1 that was enriched and isolated with iron as electron donor.

Primer	Sequence (5' → 3')	Specific for					Reference
		<i>Crenarch.</i>	<i>Euryarch.</i>	<i>Korarch.</i>	<i>Nanoarch.</i>	Strain IM1	
8aF	TCYGG TTGAT CCTGC C	+	+	+	-	-	Huber et al., 2002
8mcF	TCCCG TTGAT CCYGC GG	-	-	-	+	-	Huber et al., 2002
21F	CYGGT TGATC CYGCC RGA	+	+	NR	NR	-	DeLong, 1992
344F	AGCGG GYGCA GCAGG CGCGA	+	+	NR	NR	+	Casamayor et al., 2000
915R	GTGCT CCCCC GCCAA TTCCT	+	+	NR	NR	+	Casamayor et al., 2000
1406R	ACGGG CGGTG TGTRC AA	+	+	+	-	+	Huber et al., 2002

NR, not reported.

Strain IM1 was able to grow and produce methane with iron at a rate as high as that by the enrichment culture from which the strain had been isolated. At pH above 7.5 the strain could grow slowly with H₂ and pH optimum was 8.2. The slightly alkaliphilic characteristic of strain IM1 could be explained as an adaptation to the high pH condition in the surrounding of corroding iron.

2.4 Study of corrosion by the new methanogenic archaeon

2.4.1 Rate of methane production with metallic iron

Growth experiments to study methane production with iron by strain IM1 in more detail were carried out in glass bottles (250 ml) containing seawater medium (150 ml) and iron granules (30 g) as the source of electrons. Sterile medium with iron alone or with iron and marine hydrogen-utilizing strains *Methanococcus maripaludis* (DSMZ 2771, Jones et al., 1984), *Methanogenium organophilum* (DSMZ 3596, Widdel et al., 1989) or *Methanosarcina mazei* (DSMZ 3318, Maestrojuan et al., 1992) served as controls.

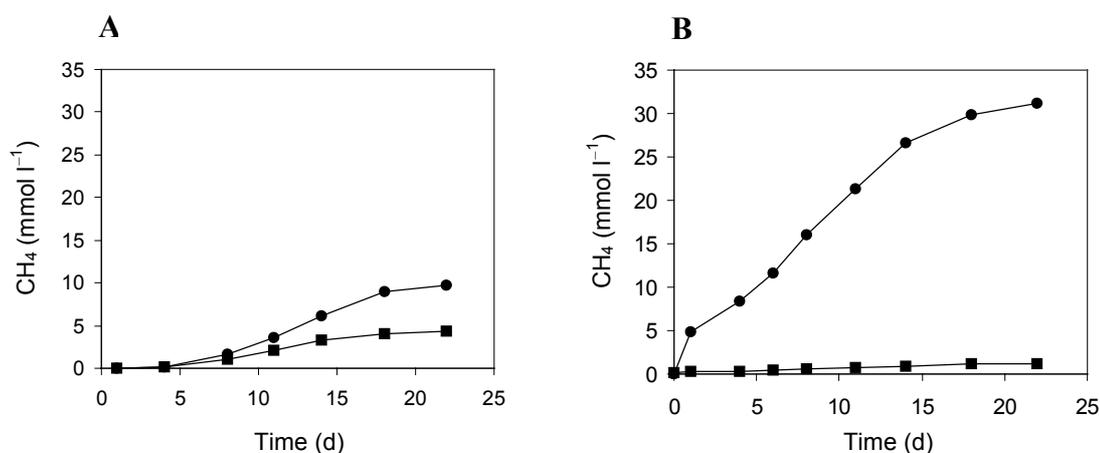


FIG. 12. Methane formation by strain IM1 (A) and the hydrogenotroph *Methanococcus maripaludis* (B) with metallic iron (■) or H₂ (●). The experiment was carried out in glass vials (250 ml) containing 150 ml seawater medium (without sulfate). Iron granules (30 g) or H₂ (added as gas mixture of H₂/CO₂ [80/20 vol./vol.] into head space of the culture tubes) were used as electron donor for CO₂ reduction. For convenience, the indicated methane is attributed exclusively to the aqueous phase.

According to the reaction of methane production from H₂ and CO₂ (reaction 9), methanogens need four mol of H₂ to produce one mol of methane. The hydrogen-utilizing strains produced methane at a rate that corresponded stoichiometrically to the rate of chemical H₂ production from iron. In contrast, strain IM1 produced methane at a considerably higher

rate than could be expected from the rate of chemical H₂ production (part II, 1, Fig. 3). With H₂, however, strain IM1 grew significantly slower than the tested H₂-utilizing strains (Fig. 12). This result provides further evidence for a more direct flow of electrons from the iron surface to the cells than via hydrogen during the process of anaerobic corrosion.

2.4.2 Hydrogen formation with metallic iron

The corrosion experiment with SRB has shown that the new type of bacterium could significantly stimulate the formation of free H₂ with iron. Analogous measurements carried out with the methanogenic strains growing with iron showed that H₂ was formed and accumulated at early growth phase of these cultures. Whereas in the culture of *Methanococcus maripaludis* H₂ was formed at a rate as low as in the sterile control, in the presence of strain IM1, H₂ formation with iron was higher (Fig. 13). After a week the process slowed down and the H₂ disappeared via consumption by the strains.

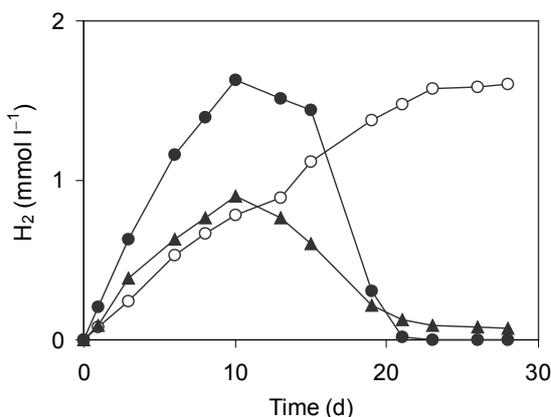


FIG. 13. Hydrogen formation in cultures of methanogens growing with metallic iron. In the culture of strain IM1 (●), a slightly increased formation of H₂ was observed, whereas in culture of the H₂-utilizing *Methanococcus maripaludis* (▲), H₂ was formed at a rate similar as in sterile control (○) and accumulated in culture bottle only at the early growth phase.

Obviously, the rate of methane production in the methanogenic enrichment culture with iron was slower than the rate of sulfate reduction in the enrichment culture of SRB with iron (Figs. 5 and 10). However, methanogenesis with iron seemed to continue constantly for a longer time than sulfate reduction with iron.

3. Proposed mechanism of corrosion by the new types of anaerobic microorganisms

Based on the obtained results, a model of anaerobic iron corrosion without involvement of hydrogen as an intermediate can be proposed. According to the new hypothesis, corrosive SRB (like strain IS4) grow in intimate contact with the iron surface can accept electrons directly from iron in an unknown manner and transfer those electrons to the sulfate reducing system (Fig. 14).

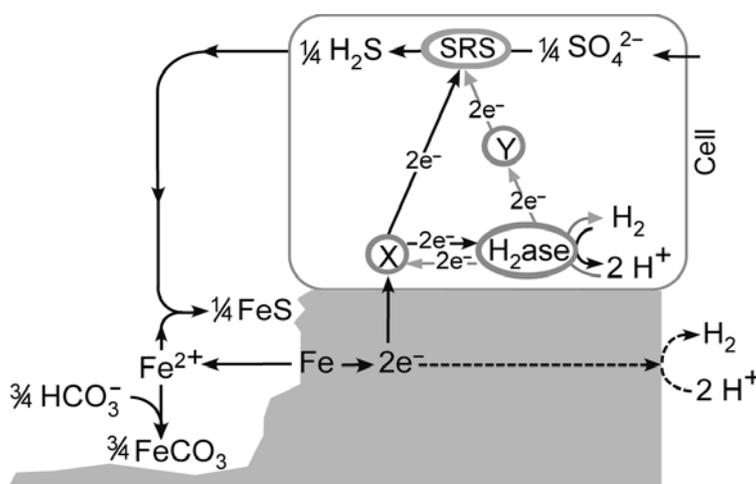


FIG. 14. Model of corrosion by the new type of sulfate-reducing bacterium. Electrons from the iron surface can be delivered to cells via cell-metal contact and used for sulfate reduction in sulfate reduction system (SRS). The involvement of a redox-active component (X) is postulated that allows electrons flow through the outer cell barrier. As a side reaction, H₂ evolution occurs via hydrogenases. There could be also an electron flux from the hydrogenases to SRS via an additional electron carrier (Y). In mixed microbial populations, the produced H₂ may be mostly scavenged by effectively H₂-utilizing microorganisms such as *Desulfovibrio* species. Corrosion by the new methanogenic strain IM1 is proposed to occur in a similar way.

There has to be an involvement of certain extracytoplasmic cell components that allow electrons to flow through the outer cell barrier as in the case of the Fe(III)-reducing components in *Geobacter* (Magnuson et al., 2000), but working in the inverse direction. On a side path, reduction of protons to H₂ via hydrogenases may occur that accounts for the observed stimulation of H₂ production with iron (see 1.5.3). This reaction is supposed to occur at the highest rate at the beginning of cell attachment to the iron surface. Such direct electron transfer from iron to hydrogenases has been recently demonstrated in experiments with NAD-dependent hydrogenase from *Rastonia eutropha* (Da Silva et al., 2002). The formation of free

H₂ at high concentration during anaerobic corrosion by strain IS4 also shows that scavenging of hydrogen cannot be the limiting step that controls the rate of corrosion by SRB. The produced H₂ in turn may serve as electron donor for H₂-utilizing microorganisms such as *Desulfovibrio* species. The new type of methanogenic archaeon (strain IM1) is proposed to corrode iron in a similar way.

In conclusion, it is likely that anaerobic corrosion under field conditions results from combination of two processes (i) a direct electron uptake by microbial cells and (ii) by chemical reaction of sulfide produced by SRB.

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Part II. Manuscripts

A Overview of the manuscripts

Results of the dissertation are presented mainly in the following manuscripts.

1. Iron corrosion by novel anaerobic microorganisms

Hang T. Dinh, Jan Kuever, Marc Mußmann, Achim Walter Hassel, Martin Stratmann, and Friedrich Widdel.

In this manuscript, mechanistic aspects of anaerobic corrosion were discussed. A direct electron uptake from iron by new types of sulfate-reducing bacteria and methanogenic archaea was shown to be the principal mechanism of anaerobic corrosion. The newly isolated microorganisms were supposed to be chiefly responsible for anaerobic corrosion under the employed conditions, and probably also *in situ*.

Concepts of this study were initiated by F. Widdel. I myself carried out microbiological experiments and part of the molecular analyses. Jan Kuever contributed with performing phylogenetic analysis. Fluorescent *in situ* hybridization was carried out by Marc Mußmann. Analyses of corroding surface were performed by Achim W. Hassel and Martin Stratmann, who also contributed with fruitful discussions, especially in the field of electrochemistry. The manuscript was prepared in editorial cooperation with F. Widdel.

2. Molecular analysis of marine sulfate-reducing bacteria enriched on corroding iron, and characterization of abundant species isolated in pure culture

Hang T. Dinh, Jan Kuever, and Friedrich Widdel.

While the first manuscript focused on the mechanism of anaerobic corrosion, this manuscript was devoted to investigate diversity of “corrosive” sulfate-reducing bacteria in our laboratory corrosion model, i.e. enrichment cultures with metallic iron as the only electron donor for sulfate reduction. The newly isolated strains of sulfate-reducing bacteria were characterized in detail and compared with conventional SRB originally isolated with common substrates such as lactate and hydrogen.

Concepts of this study were developed in cooperation with F. Widdel. Microbiological experiments and molecular analyses were accomplished by myself. Jan Kuever contributed with performing phylogenetic analysis and valuable discussions. The manuscript was prepared in editorial cooperation with F. Widdel.

B Manuscripts

1

Iron corrosion by novel anaerobic microorganisms

Hang T. Dinh ¹, Jan Kuever², Marc Mußmann¹, Achim Walter Hassel³,
Martin Stratmann³ & Friedrich Widdel^{1*}

Manuscript submitted

¹ Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, 28359 Bremen, Germany

² Institute for Material Testing, Paul-Feller-Str. 1, 28199 Bremen, Germany

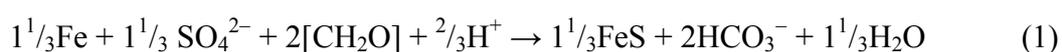
³ Max Planck Institute for Iron Research, Max-Planck-Strasse 1, 40237 Düsseldorf, Germany

*** Corresponding author.**

Corrosion of iron presents a serious economic problem (Corrosion doctors, www.corrosion-doctors.org). Whereas aerobic corrosion is primarily a chemical process (Corrosion doctors, www.corrosion-doctors.org; Uhlig, 1985), anaerobic corrosion is frequently linked to the activity of sulphate-reducing bacteria (SRB) (Pankhania, 1988; Widdel, 1992; Lee et al., 1995; Cord-Ruwisch, 2000; Hamilton, 2003). SRB are supposed to act upon iron by produced hydrogen sulphide as a corrosive agent (Costello, 1974; Widdel, 1992; Lee et al., 1995;), and by consumption of "cathodic" hydrogen formed on the metal with protons from water (von Wolzogen Kuehr and van der Vlugt, 1934; Pankhania, 1988; Widdel, 1992; Lee et al., 1995; Cord-Ruwisch, 2000; Hamilton, 2003). Among SRB, *Desulfovibrio* species with their capacity to effectively consume hydrogen are conventionally regarded as main culprits of anaerobic corrosion (von Wolzogen Kuehr and van der Vlugt, 1934; Booth and Tiller, 1968; Pankhania et al., 1986; Pankhania, 1988; Widdel, 1992; Lee et al., 1995; Cord-Ruwisch, 2000; Hamilton, 2003). However, the biological mechanism behind anaerobic corrosion is 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 known hydrogen-utilizing methanogens, again suggesting a more direct access to electrons from the metal than via hydrogen consumption.

Some 10% of all corrosion damages to metals and non-metals may result from microbial activities (Iverson and Olson, 1984). A significant process in this respect is the anaerobic corrosion of iron or steel, for instance in oil and gas technology (Iverson and Olson, 1984; Lee et al., 1995; Cord-Ruwisch, 2000). The primary oxidation reaction of iron ($\text{Fe} \rightleftharpoons \text{Fe}^{2+} + 2\text{e}^-$; $E^0 = -0.44 \text{ V}$) can be driven by numerous electron acceptors. In oxic humid surroundings, the abiotic reaction with O_2 ($E^0_{\text{pH}=7} = +0.82 \text{ V}$) leads to rust (Uhlig, 1985). In anoxic surroundings, H^+ ions from water serve as electron acceptors and yield H_2 ($E^0_{\text{pH}=7} = -0.41 \text{ V}$). Of the individual steps ($2\text{e}^- + 2\text{H}^+ \rightarrow 2\text{H}_{(\text{adsorbed})} \rightarrow \text{H}_{2(\text{adsorbed})} \rightarrow \text{H}_{2(\text{aqueous})}$), the combination of the H atoms is assumed to be kinetically hampered (Bockris and Reddy, 1970) such that the overall process is very slow on iron in anoxic, sterile water. However, sulphate-reducing bacteria (SRB) stimulate anaerobic oxidation of iron enormously (Pankhania, 1988; Widdel, 1992; Lee et al., 1995; Cord-Ruwisch, 2000; Hamilton, 2003). Anaerobic corrosion by SRB often takes place under biofilms and tends to pit the metal (Beech et al., 1994; Lee et al. 1995; Hamilton, 2003). An indirect and a direct corrosion mechanism are distinguished (Widdel, 1992; Lee et al., 1995); these may occur simultaneously at different extent, depending on the load of waters with biodegradable organic compounds.

The indirect mechanism is a chemical attack of hydrogen sulphide ($\text{Fe} + \text{H}_2\text{S} \rightarrow \text{FeS} + \text{H}_2$) which is faster than that of pure water on iron (Widdel, 1992; Lee et al., 1995; Cord-Ruwisch, 2000). Because SRB commonly use organic compounds (approximate bulk formula usually $[\text{CH}_2\text{O}]$) and often also H_2 for sulphate reduction, the net reaction of indirect corrosion (here for SRB with complete oxidation of organic compounds) can be written as:



In the direct mechanism according to the depolarisation theory (von Wolzogen Kuehr and van der Vlugt, 1934), SRB are supposed to stimulate corrosion by scavenging of the "cathodic hydrogen" formed on water-exposed iron. However, it has remained unclear whether the scavenged form is free H_2 or a precursor. Irrespective of this, the resulting net reaction of direct corrosion (with precipitation of sulphide) is:



In bicarbonate-rich waters, Fe^{2+} ions may yield FeCO_3 . The direct corrosion mechanism is commonly attributed to *Desulfovibrio* species (von Wolzogen Kuehr and van der Vlugt, 1934; Booth and Tiller, 1968; Pankhania et al., 1986; Pankhania, 1988; Widdel, 1992; Beech et al., 1994; Lee et al., 1995; Cord-Ruwisch, 2000), the best-studied SRB. Their efficient H_2

utilization (Widdel and Bak, 1992; Rabus et al., 2000) has often favoured the view that acceleration of anaerobic corrosion is due to this capacity. Indeed, stimulating effects of *Desulfovibrio* cells on the current via iron cathodes have been observed (Booth and Tiller, 1968; Pankhania et al., 1986). On the other hand, stimulation of iron oxidation due to H₂ consumption has been questioned (Hardy, 1983; Widdel, 1992; Cord-Ruwisch, 2000); for instance, H₂ did not inhibit its own formation on iron in neutral water (Cord-Ruwisch, 2000). It is true that *Desulfovibrio* species (Cord-Ruwisch and Widdel, 1986; Pankhania et al., 1986; Pankhania, 1988); and also methanogenic archaea (Daniels et al., 1987; Deckena and Blotevogel, 1992) formed sulphide or methane, respectively, with metallic iron in growth media; however, this was probably a secondary consumption of chemically formed H₂, without stimulation of corrosion (Deckena and Blotevogel, 1992; Cord-Ruwisch, 2000).

To search for possibly yet undetected SRB with potential for direct corrosion (equation (1)), we established enrichment cultures with iron specimens as the only electron donor and marine sediment as source inoculum. Iron has been used as a reductant in a former anaerobic enrichment technique (Schlegel, 1993), but has not been reported to yield cultures other than those of *Desulfovibrio* species growing with organic substrates or H₂. For comparison, we enriched parallel cultures with H₂ instead of iron. Within two weeks, sulphate reduction in cultures with iron exceeded the endogenous sulphate reduction in the sediment that is obvious in iron- and H₂-free controls (4 mM versus 2 mM). Since corrosive SRB may associate with the metal, subcultures with fresh iron were inoculated with a part of the previous iron specimens. As carbon source, cultures contained either CO₂ alone, or CO₂ plus acetate (1 mM). Sulphate reduction in subcultures became faster, and formation of black ferrous sulphide became visible. Microscopy revealed only few free-living cells. In contrast, the enrichment culture with H₂ yielded abundant cells in the free medium.

Two strains, IS4 and IS5, were isolated from iron-grown sulphate-reducing enrichment cultures (without or with acetate, respectively). A third strain, HS2, was isolated from the H₂-grown enrichment culture (with acetate). Strain IS4 is rod-shaped (Fig. 1) and affiliates with *Desulfobacterium* species, whereas the other two strains are comma-shaped (not shown) and represent *Desulfovibrio* species (Fig. 2a). All three strains grew also by sulphate reduction with lactate or H₂. With H₂ and CO₂, strain IS4 did not depend on an organic carbon source (lithoautotrophic growth), whereas strains IS5 and HS2 needed acetate for cell synthesis (lithoheterotrophic growth). Acetate was not oxidized. With lactate or H₂, strain IS4 exhibited much slower growth (doubling time, 2–3 days) than strains IS5, HS2 and the authentic species, *Desulfovibrio salexigens* and *D. vulgaris* (doubling time, ≤1 day).

A fluorescent 16S rRNA-targeted oligonucleotide probe specifically designed for strain IS4 revealed high numbers of corrosion product-associated cells in the enrichment culture with similar shape as strain IS4 (Fig. 1d, e). These cells represented the majority of all detectable cells. A common oligonucleotide probe for *Desulfovibrio* species (Manz et al., 1998) did not reveal hybridisation.

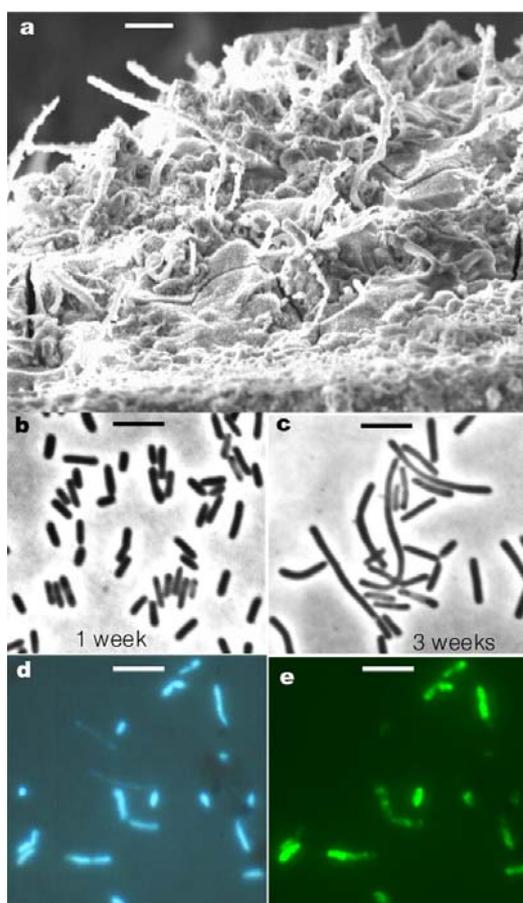


FIG. 1. Microscopy of cultures. **a**, Scanning electron micrograph of an iron coupon incubated with strain IS4 for seven weeks. Filamentous cells are embedded in precipitated FeCO_3 and FeS . **b**, Phase contrast micrographs of viable cells of strain IS4 grown for 1 week with lactate. **c**, Cells from the same culture after three weeks. **d**, General (DAPI) staining of cells in an FeS particle from the metal surface in an enrichment culture with metallic iron. **e**, The same section with visualisation of specific fluorescent staining with an oligonucleotide probe designed for the *Desulfobacterium*-like strain IS4. Bars, 10 μm .

We also attempted the enrichment of methanogenic marine microorganisms with metallic iron in low-sulphate medium. Iron-dependent methane production became first 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 so far yielded only a

shortened fragment (1000 bp); this revealed an affiliation with *Methanobacterium* or *Methanobrevibacter* (Fig. 2b). In the absence of metallic iron, strain IM1 could slowly grow with H₂ + CO₂ if the pH was above 7.5.

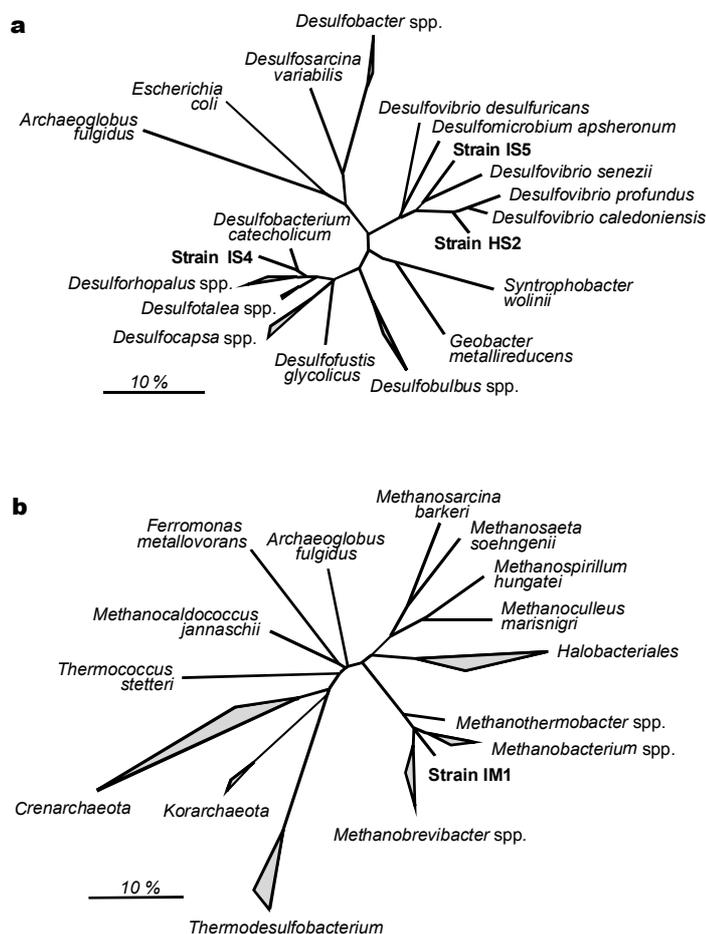


FIG. 2. Phylogenetic trees based on 16S rRNA gene sequences from newly isolated pure cultures of anaerobic marine microorganisms. **a**, Relationships of SRB isolated from enrichment cultures with metallic iron (strains IS4 and IS5) or hydrogen (strain HS2) and sulphate. **b**, Relationships of a methanogenic archaeon (strain IM1) isolated from an enrichment culture with metallic iron and very low sulphate concentration. Bars indicate 10% estimated sequence divergence.

As a direct measure of corrosiveness, we measured sulphate reduction or methanogenesis with iron as the only electron donor by the newly isolated as well as by authenticated species of SRB and methanogenic archaea, respectively (Fig. 3a, b). The authenticated species are known hydrogen utilisers. Sulphate reduction to sulphide by strain IS4 with iron was fast, similar as in the enrichment culture. Sulphate reduction slowed down with time, but became faster again if fresh iron was added (not shown), indicating that formed surface layers (Fig. 1a) present a certain barrier. Sulphate reduction by strain IS5 was slightly slower than by strain IS4. In comparison, sulphate reduction by strain HS2 and the authentic

species *D. salexigens* and *D. vulgaris* was rather marginal and the rate is in agreement with a consumption of the chemically formed H_2 (4 mol H_2 reducing 1 mol sulphate). Methanogenesis with iron was also more pronounced with the new strain IM1 than with the authenticated species *Methanogenium maripaludis* (Fig. 3b), *Methanococcus organophilum* and *Methanosarcina mazei* (not shown). The rate of methanogenesis with iron by the authenticated species is again in accordance with consumption of the chemically formed H_2 (4 mol H_2 needed for 1 mol CH_4).

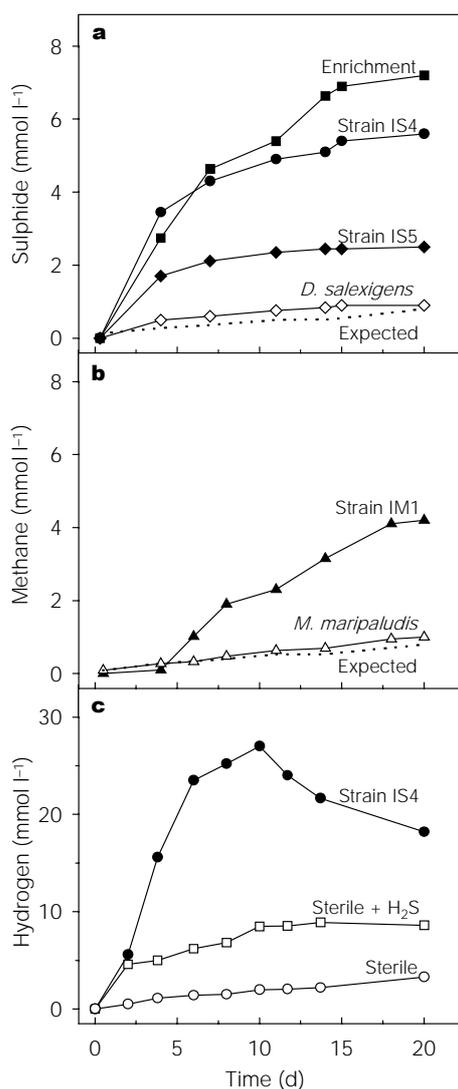


FIG. 3. Incubation experiments with iron granules (30 g in 150 ml medium) as sole electron donor. **a**, Sulphide formation (calculated via sulphate consumption) by strains IS4, IS5 and *Desulfovibrio salexigens*, and the original enrichment culture. **b**, Methane formation by strain IM1 and *Methanococcus maripaludis*. **c**, Hydrogen formation by strain IS4, and in sterile incubations with iron in the absence or presence of hydrogen sulphide (4 mM). In the latter, hydrogen production became slower after binding of free sulphide as FeS. The expected sulphate reduction or methanogenesis by consumption of the chemically formed hydrogen is also indicated (panels a, b).

Since the speed of sulphate reduction by strains IS4 and IS5 and of methanogenesis by strain IM1 with iron as the only electron donor cannot be explained by consumption of the chemically formed hydrogen, these organisms have to obtain reducing equivalents from the metal in a more efficient manner than the other hydrogen-consuming microorganisms tested. Iron-dependent growth of strains IS4 even led to an accumulation of H₂ (Fig. 3c) which, therefore, cannot be the rate-limiting intermediate.

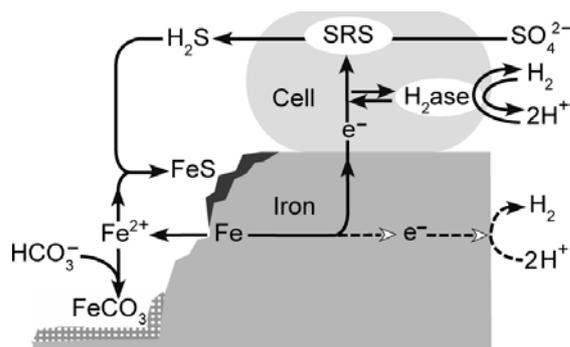


FIG. 4. Suggested electron flow during anaerobic corrosion by the *Desulfobacterium*-like strain IS4. Complete stoichiometric equations (see equation (2), and Methods) and cellular structures (cytoplasmic membrane, cell wall etc.) are not depicted. Hydrogen formed at the beginning with fresh metallic iron (Fig. 3c) is explained as a side reaction ("overflow") of a branched electron transport system involving hydrogenase (H₂ase), and by chemical formation. An analogous direct electron flow with CO₂ being reduced to CH₄ may occur in the novel methanogenic isolate, strain IM1.

A hypothesis for an efficient, direct use of metallic iron for sulphate-reduction or methanogenesis would be an electron uptake via cell-surface associated redox proteins (Fig. 4). The principle of an electron uptake via a peripheral redox protein is known from acidophilic aerobic bacteria that oxidise dissolved ferrous to ferric iron (Appia-Ayme et al., 1999). The inverse process, a delivery of electrons (from the metabolism of organic substrates) to insoluble (solid) external surfaces occurs in iron(III)-reducing bacteria (Bond and Lovley, 2003). In fractionated cell extract of *D. vulgaris*, an outer membrane-associated cytochrome was shown to be reduced by metallic iron (von Ommen Kloeke et al., 1995). In the present study, however, the same *D. vulgaris* strain did not reveal a corrosive potential like strain IS4. Nevertheless, such an outer membrane component must be also postulated as part of an effective electron transfer chain to the sulphate-reducing system in strains IS4 and IS5 growing with iron. The direct electron flow would shortcut electron utilisation via H₂ the formation of which involves the slow combination of H-atoms (Bokris and Reddy, 1970). The observed hydrogen (Fig. 3c) may be formed via a side-path involving hydrogenase, and via

slower chemical reaction. The hydrogenase may otherwise function in growth with external hydrogen as a substrate. Detailed hypotheses of electron flow are presently not possible because knowledge of the topology and function of redox proteins in different SRB and methanogenic archaea is insufficient (Rabus et al., 2000; Deppenmeier, 2002).

The abundance of cells resembling strain IS4 in our iron-corroding enrichment culture and the effective iron-dependent sulphate reduction by this strain suggests an important, so far overlooked role of such or similar bacterial types in anaerobic corrosion. Proof of this assumption requires the *in situ* examination of sites with corroding iron or steel. The natural significance of the capacity for the use of metallic iron for sulphate reduction is unknown. Apart from rare meteorites, metallic iron as a technical product represents a very recent growth substrate on an evolutionary time scale. One may speculate that iron-utilising anaerobes can also obtain electrons in direct contact with certain other microorganisms.

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 (Widdel and Bak, 1992) with 28 mM sulphate (0.1 mM for methanogens) in butyl rubber-stoppered 200 ml-bottles or 20 ml-tubes under an atmosphere (half of liquid volume) of N₂ + CO₂ (90/10, vol./vol.). If indicated, 1 mM sodium acetate was added as a carbon source in addition to the routinely added bicarbonate. 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 source of electrons. For subculturing, 10% of the culture liquid and part of the iron specimens were transferred every seven weeks. For control, parallel enrichment cultures were carried out with H₂ + CO₂ (80/20, vol./vol.) and 1 mM acetate (carbon source) without metallic iron.

For strain isolation, precipitates from the iron surface in enrichment cultures were homogenised anaerobically and serially diluted (Widdel and Bak, 1992) in medium with iron granules. The highest dilutions showing sulphate reduction or methanogenesis were again diluted. SRB were finally diluted in anoxic agar with a mixture of lactate, propionate, butyrate, pyruvate, ethanol (each 2 mM) and hydrogen (Widdel and Bak, 1992). Colonies were transferred to liquid medium with iron.

Authenticated strains were obtained from the German Collection of Microorganisms (Braunschweig, Germany).

Chemical analyses

Sulphate was determined micro-gravimetrically as washed, dried BaSO₄ precipitated from 1 ml samples with the same volume of 0.2 M BaCl₂ and 0.2 M HCl. H₂ and CH₄ were quantified on a gas chromatograph with a thermal conductivity or flame ionisation detector, respectively. Minerals on the iron surface were analysed by X-ray photoelectron spectroscopy.

Analyses of 16S rRNA genes

DNA from SRB was extracted with the DNeasy Tissue kit (Qiagen). Nearly full-length (1500 bp) 16S rRNA gene sequences from SRB were amplified using general bacterial primers (Sahm et al. 1999). DNA from the methanogenic isolate was retrieved via freeze-thaw, detergent and proteinase treatment (Sahm et al., 1999); a partial (about 1000 bp) 16S rRNA gene sequence was amplified with archaeal primers (Huber et al., 2002). PCR products were purified with the Quiaquick Spin PCR purification kit (Qiagen). Determined sequences were added to the rRNA gene sequence database of Technical University Munich by using the ARB program package (Strunk and Ludwig, www.mikro.biologie.tu-muenchen.de). A phylogenetic tree was constructed via maximum parsimony, neighbor joining and maximum likelihood analyses. The partial sequence from the methanogenic isolate was inserted into the constructed tree according to parsimony criteria without affecting the overall tree topology.

Specific cell hybridisation and unspecific staining

Particles collected from corroding 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 filtered on polycarbonate filters (pore size, 0.2 µm; Millipore). A horseradish peroxidase-labelled probe (5'-CTCCTCCTGCTGCAGTAGCT-3') was specifically designed and synthesised (Thermohybrid) for strain IS4. After hybridisation at 35°C in the presence of 55% (vol./vol.) formamide and washing, the reaction with the dye (tyramide-Cy3) was performed (Pernthaler et al., 2002). General cell staining was performed with the standard DAPI (4',6-diamidino-2-phenylindole) technique.

Further equations

Indicated redox potentials are versus standard hydrogen electrode ($E^0 = 0$ V).

Electrons from iron may not only reduce protons, but also water directly to hydrogen, which is especially relevant at high pH.

The equation $2e^- + 2H_2S \rightarrow 2HS^- + H_2$ presumably represents a rapid step in the chemical reaction of hydrogen sulfide with metallic iron (Costello, 1974).

Considerations underlying equation (1) include equations for sulphate reduction with the organic compound ($SO_4^{2-} + 2[CH_2O] \rightarrow H_2S + 2HCO_3^-$) and with hydrogen ($SO_4^{2-} + 4H_2 + 2H^+ \rightarrow H_2S + 4H_2O$). If SRB reduce 1 mol SO_4^{2-} with an organic compound to 1 mol H_2S , the latter yields 1 mol H_2 by chemical reaction with iron. Utilisation of H_2 for further sulphate reduction yields $\frac{1}{4}$ mol H_2S which then leads to $\frac{1}{4}$ mol H_2 . Continuation ad infinitum leads to a total amount of $1\frac{1}{3}$ (sum of infinite row $1 + \frac{1}{4} + \frac{1}{16}$ etc.) mol H_2S that attacks the iron.

In the presence of bicarbonate, the direct corrosion leads to ferrous carbonate as additional precipitate, according to $4Fe + SO_4^{2-} + 3HCO_3^- + 5H^+ \rightarrow FeS + 3FeCO_3 + 4H_2O$. With methanogenesis, the equation including carbonate precipitation is $4Fe + 5HCO_3^- + 5H^+ \rightarrow CH_4 + 4FeCO_3 + 3H_2O$.

Further aspects of corrosion mechanisms such as H_2 -induced metal cracking (especially promoted by H_2S) (Corrosion doctors, www.corrosion-doctors.org; Uhlig, 1985), or enhanced corrosion in environments with intermittent oxic and anoxic conditions (Widdel, 1992) have not been included.

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Correspondence and request for materials should be addressed to F.W. (e-mail: 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).

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Molecular analysis of marine sulfate-reducing bacteria enriched on corroding iron, and characterization of abundant species in pure culture

Hang T. Dinh,¹ Jan Kuever,² and Friedrich Widdel^{1*}

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¹ Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany

² Institute for Material Testing/Foundation Institute for Material Science, Paul-Feller-Str. 1, 28199 Bremen, Germany

*** Corresponding author.**

A marine enrichment culture with metallic iron as the only electron donor for bacterial sulfate reduction was investigated as an experimental model for anaerobic biocorrosion. Analysis of 16S rRNA genes from the enrichment culture by PCR and denaturing gradient gel electrophoresis (DGGE) revealed a characteristic fragment affiliating with the *Desulfobacterium* cluster (Deltaproteobacteria). This fragment was not observed in parallel enrichment cultures (controls) with H₂ as electron donor instead of iron and is, therefore, assumed to represent sulfate-reducing bacteria that play a crucial role in anaerobic iron corrosion. The iron-grown enrichment culture revealed in addition a specific line of descent in the *Desulfovibrio* cluster, probably representing a minor population. The H₂-grown enrichment cultures revealed different *Desulfovibrio* species. A rod-shaped isolate, strain IS4, and a vibrioid isolate, strain IS5, were characterized as representatives of the *Desulfobacterium*- and *Desulfovibrio*-related populations, respectively, in the iron-grown enrichment cultures. With metallic iron as a source of electrons, these isolates, in particular strain IS4, exhibited faster growth than conventional *Desulfovibrio* species. Strains IS4 and IS5 could also oxidize H₂, formate, lactate or pyruvate; the latter two compounds were incompletely oxidized to acetate. Based on 16S rRNA gene sequences, the closest cultivated relatives of strains IS4 and IS5 are *Desulfobacterium catecholicum* and *Desulfovibrio senezii*, respectively (sequence similarity, 95 and 92%, respectively). Strains IS4 and IS5 are regarded as new, corrosive species of sulfate-reducing bacteria; the names *Desulfobacterium corrodens* and *Desulfovibrio ferrophilus*, respectively, are proposed.

Whereas aerobic iron corrosion (rusting) is mostly an abiotic process (Uhlig, 1985), anaerobic iron corrosion is catalyzed to a large extent by microorganisms, in particular by sulfate-reducing bacteria (Hamilton, 1985; Widdel, 1992; Lee et al., 1995; Cord-Ruwisch, 2000). Anaerobic corrosion may cause severe damage to steel constructions in the production, transport and storage of oil which is frequently connected with the presence of seawater or other sulfate-rich salt-waters (Hamilton, 1985; Cord-Ruwisch et al., 1987; Cord-Ruwisch, 2000). Seawater with its naturally high sulfate concentration (28 mM) favours the development of sulfate-reducing bacteria as a dominant group of anaerobic degraders (Widdel and Bak, 1992; Rabus et al., 2000).

Two principal mechanisms, an indirect and a direct one, may be distinguished by which sulfate-reducing bacteria corrode iron (Widdel, 1992; Lee et al., 1995). Both mechanisms may occur simultaneously at different extent, depending on the organic load of the water system.

The indirect mechanism is a purely chemical attack of H₂S as a corrosive agent on iron (according to $\text{Fe} + \text{H}_2\text{S} \rightarrow \text{FeS} + \text{H}_2$) (Costello, 1974; Widdel, 1992; Lee et al., 1995) and can thus be caused by all types of organotrophically growing sulfate-reducing bacteria. The chemically formed H₂ may be also used as electron donor (for details of equations see reference Dinh et al., 2003).

The direct mechanism of anaerobic corrosion has been formulated first in the classical cathodic depolarization theory (Wolzogen Kuehr and van der Vlugt 1934). According to this, electrons from the primary redox reaction of iron ($\text{Fe} \rightleftharpoons \text{Fe}^{2+} + 2 \text{e}^-$; $E^0 = -0.44 \text{ V}$) reduce protons from water (or water directly) to form H₂ that is immediately utilized by sulfate-reducing bacteria; the scavenge of this "cathodic hydrogen" (in textbooks sometimes regarded as "protective hydrogen layer") is believed to stimulate the net oxidation, viz. corrosion of iron. However, the actual chemical state of the scavenged hydrogen has not been elucidated, and unspecific equations are often preferred to formulate its chemical formation ($2 \text{H}^+ + 2 \text{e}^- \rightarrow 2 [\text{H}]$) and subsequent biological utilization ($8 [\text{H}] + \text{SO}_4^{2-} + 2 \text{H}^+ \rightarrow \text{H}_2\text{S} + 4 \text{H}_2\text{O}$). With the reaction of hydrogen sulfide with ferrous iron to insoluble FeS, the resulting overall equation of the direct corrosion mechanism is $4 \text{Fe} + \text{SO}_4^{2-} + 8 \text{H}^+ \rightarrow 3 \text{Fe}^{2+} + \text{FeS} + 4 \text{H}_2\text{O}$.

The direct corrosion mechanism is conventionally attributed to members of a single genus, *Desulfovibrio*, which are the longest-known and most easily cultivated sulfate-reducing bacteria (Postgate, 1984; Widdel and Bak, 1992). Also experimental studies of corrosion by sulfate-reducing bacteria have been consistently carried out with *Desulfovibrio* species (Iverson, 1966; Booth and Tiller, 1968; Hardy, 1983; Cord-Ruwisch and Widdel, 1986; Pankhania, 1986; Beech et al., 1994). Their common capacity to grow well with free

(dissolved) H₂ has been in favour of the view that anaerobic corrosion is caused by the scavenge of this compound (for a review see reference Pankhania, 1988). On the other hand, experiments and arguments from a physicochemical point of view have raised doubts whether scavenge of free H₂ can stimulate iron oxidation in aqueous surrounding (Hardy, 1983; Widdel, 1992; Cord-Ruwisch, 2000). A thermodynamic equilibrium shift is not needed for anaerobic iron oxidation to proceed; at the usually low Fe²⁺ concentrations *in situ*, electrons from metallic iron (usually more negative than -0.5 V) can form H₂ ($E^{0'} = -0.41$ V) in an exergonic reaction (Widdel, 1992). Also from a kinetic point of view, stimulation by H₂ consumption is unlikely; among the steps leading to H₂ ($2e^- + 2H^+ \rightarrow 2 H_{(adsorbed)} \rightarrow H_{2(adsorbed)} \rightarrow H_{2(aqueous)}$), the combination of the H-atoms on metallic iron is probably the rate-controlling reaction (Bockriss and Reddy, 1970) and therefore expected to be independent of a bacterial consumption of free (dissolved) H₂ (Widdel, 1992).

The demonstration that metallic iron can provide reducing equivalents to hydrogenotrophic species of sulfate-reducing bacteria (Iverson, 1966; Cord-Ruwisch and Widdel, 1986; Pankhania et al., 1986) or methanogenic archaea (Daniels, 1987; Deckena and Blotevogel, 1990) for the reduction of sulfate or CO₂, respectively, does not necessarily prove that anaerobic corrosion is due to a scavenge of hydrogen. It is likely that H₂ from slow chemical reaction (net reaction: $Fe + 2 H^+ \rightarrow Fe^{2+} + H_2$) was utilized in a secondary process without stimulation of corrosion (Deckena and Blotevogel, 1992; Widdel, 1992; Cord-Ruwisch, 2000).

To reinvestigate the potential corrosiveness of anaerobic microorganisms, we recently enriched sulfate-reducing bacteria from marine samples directly with metallic iron as the only electron donor (Dinh et al., 2003). Continued transfer of iron specimens and the use of surface-associated layers for dilution series with metallic iron resulted in the isolation of novel type of sulfate-reducing bacterium, the *Desulfobacterium*-like strain IS4. This reduced sulfate with iron at much higher rate than conventional *Desulfovibrio* species (isolated with H₂ or lactate). Such high rates could not be accounted for by the chemically formed H₂ as electron donor. It was, therefore, suggested that this strain stimulated corrosion by a direct scavenge of electrons from iron, possibly through extracytoplasmic redox proteins, and thus circumvented the slow chemical formation of H₂ on iron. A second isolate, the novel *Desulfovibrio*-like strain IS5, reduced sulfate with iron less rapidly, but still faster than conventional *Desulfovibrio* species. SRB as represented by the new isolates can be assumed to play an important role in anaerobic corrosion also under non-axenic conditions and *in situ*. To further substantiate this assumption, we analyzed in the present study whether the original iron-grown

enrichment culture indeed contained a unique, distinct population in comparison to an enrichment culture with H₂. Furthermore, we present a more detailed characterization and classification of the newly isolated strains IS4 and IS5. Diagnostic characteristics can be of applied interest, e.g. in future monitoring and identification of such types of SRB in corroding systems. "Conventional" *Desulfovibrio* species isolated with H₂ or lactate were included for comparison.

Materials and methods

Sources of bacteria. One set of enrichment cultures was obtained from anoxic (black) subsurface sediment in the tidal area (wadden sea) of the North Sea near Wilhelmshaven (Germany) as described (Dinh et al., 2003). This was also the source of the sulfate-reducing strains IS4, IS5 and HS2. A further enrichment culture was established in the present study with anoxic sediment from Halong Bay (Vietnam). *Desulfovibrio salexigens* (DSM 2638^T) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Cultivation. Techniques for preparation of anoxic medium, isolation and cultivation of SRB were as described previously (Widdel and Bak, 1992). Briefly, cultures were grown in HCO₃⁻/CO₂ buffered, artificial seawater medium with 1 mM sulfide as reductant and 28 mM sulfate. The medium was supplemented with a non-chelated solution of trace elements and defined mixtures of vitamins (Widdel and Bak, 1992). Cultures were grown in bottles (200 ml) or tubes (20 ml) filled with anoxic medium to 2/3 of the total volume and sealed with butyl-rubber septa under an atmosphere of N₂-CO₂ (90/10, vol/vol). If indicated, 1 mM sodium acetate was added as organic carbon source. In the enrichment cultures, iron wire (diameter 1.2 mm; Alpha, Kalsruhe, Germany) and iron coupons (0.1 × 1 × 6 cm) were used as the source of electrons. Before use, the iron was degreased in acetone, freed from oxidic surface layers by immersion in 2 M HCl for 5 min, washed several times with sterile anoxic water, and finally dried in an N₂ atmosphere. Cultures were incubated at 28 °C in the dark. The size of inocula for the initial enrichment (added sediment samples) and subsequent transfers was 10% (vol/vol). Transfers of cultures were performed every 6 to 8 weeks inside an anaerobic chamber. In addition to liquid aliquots, also a part of the iron specimens was transferred to the fresh medium. For control, parallel enrichment cultures were carried out with H₂ as electron donor instead of iron under otherwise identical conditions. To enrich SRB

with H₂ at sulfate reduction rates comparable to the relatively low ones with iron, H₂ was slowly provided via diffusion through a silicon membrane (Fig. 1).

For maintenance, the isolated SRB were grown on lactate (20 mM) or under H₂-CO₂ (80/20, vol/vol), stored at 4 °C and transferred every 8 weeks. For purity control, cultures were transferred to Marine Broth 2216 (Difco, Heidelberg, Germany) containing 28 mM sulfate and examined microscopically.

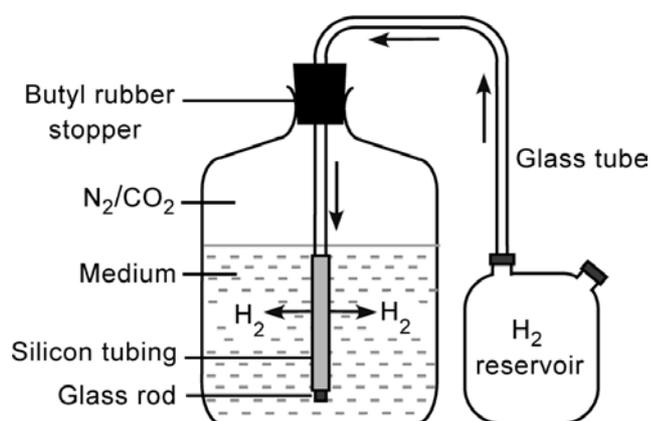


FIG. 1. Device used for the enrichment of sulfate-reducing bacteria with H₂ provided at low rate. H₂ from a reservoir was slowly delivered to the bacteria (culture volume, 80 ml) via diffusion through a piece of silicon tube (length, 15 mm; diameter, 8 mm; thickness, 1.5 mm) with a closed end.

Physiological studies. Bacterial growth was monitored turbidimetrically at 660 nm. Electron donor and acceptor tests were carried out at concentrations of 0.5 mM for catechol, 2 mM for benzoate and sulfite, 5 mM for amino acids, and 10 mM for all other compounds mentioned; sodium salts were used throughout. Optimum growth temperatures were determined in a thermal-block with a temperature gradient ranging from 10 °C to 45 °C. For the study of the pH range for growth, a saltwater medium with a lower magnesium and calcium ion concentration (Widdel and Bak, 1992) than in the routinely used artificial seawater medium was used to minimize precipitation of carbonates and phosphates at high pH values. The medium was adjusted to desired pH values with sterile Na₂CO₃ or H₂SO₄ solution. The salt requirement was examined in the artificial seawater medium (480 mM Na⁺, 56 mM Mg²⁺), in brackish medium (here a 1:1 mixture of seawater and freshwater medium), and freshwater medium (50 mM Na⁺, 2 mM Mg²⁺). All experiments were performed in duplicate. Bacterial growth was always recorded after the second transfer under the same conditions.

Microscopy. Cells in the culture medium were examined by phase-contrast microscopy. Cells in dark sulfidic precipitates were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI) (Velji and Albright, 1993).

Chemical analyses. Sulfate was determined as BaSO₄ precipitated in a gravimetric microassay (Nauhaus et al., 2002). Sulfide was quantified photometrically as colloidal CuS (Cord-Ruwisch, 1985). Organic acids in aqueous medium were quantified on a high-performance liquid chromatography (HPLC) system (Sykam, Eresing, Germany) equipped with an SS-100-H⁺ ion exclusion column (7.8 × 300 mm; Sierra Separation, Nevada, USA). The eluent was 5 mM H₂SO₄ at a flow rate of 0.6 ml min⁻¹. The column temperature was 60 °C. Eluting peaks were detected by their absorption at 210 nm in an S 3204 UV-detector (Linear Instruments, Nevada, USA). Prior to HPLC analysis, samples (1 ml) were centrifuged and filtered through HPLC filters (Minisart RC4, pore size 0.2 µm; Satorius, Göttingen, Germany).

For determination of the guanine plus cytosine (G+C) content, DNA was purified via hydroxyapatite (Cashion et al. 1977), hydrolyzed and analyzed by HPLC (Mesbah et al. 1989). The analysis was carried out at the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany.

DGGE analysis. Total DNA from the enrichment cultures was retrieved after freeze-thawing cycles with detergent and proteinase K treatment as described previously (Sahm et al., 1999). The universal PCR primer pair GM5F and 907R specific for the domain *Bacteria* was used to amplify 550 bp fragments of the 16S rRNA gene (nucleotide positions 357–907, corresponding to *Escherichia coli* numbering) (Muyzer et al., 1995). A GC-rich clamp was added to 5'-end of the forward primer (GM5F) to stabilize the melting PCR products on DGGE gels (Muyzer et al., 1995). PCR was carried out in touchdown mode with the annealing temperature decreasing in 20 cycles from 65 to 55 °C; at the latter temperature, PCR was extended for further 16 cycles. Bovine serum albumin (BSA; Sigma, Steinheim, Germany) was added to the PCR solution (final concentration 0.3 mg ml⁻¹) to prevent inhibition of the polymerase by humic substances.

DGGE analysis of PCR amplified 16S rRNA gene fragments was performed by using the D-GeneTM system (Bio-Rad Laboratories, Munich, Germany) as described previously (Muyzer et al, 1998). PCR products were separated in 6% (wt/vol) polyacrylamide gels of 1 mm thickness with a 20–70% gradient of denaturant in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3). Electrophoresis was performed at a constant voltage of

100 V (gel length, 16 cm) for 20 h at a temperature of 60 °C. After electrophoresis, the gels were stained with ethidium bromide (0.5 µg ml⁻¹) and photographed on an UV (302 nm) transillumination table. Prominent bands from the DGGE gels were excised and the DNA was eluted for 12 h in 100 µl water at 4 °C. The eluted DNA was reamplified with the primers GM5F (without GC-clamp) and 907R and sequenced. In some cases, reamplified products of the DGGE bands were cloned in *E. coli* prior to sequencing. For this purpose, the TOPO TA Cloning system with the pCR2.1-TOPO cloning vector (Invitrogen, Kalsruhe, Germany) was used. Plasmids were recovered using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The inserts were sequenced with primer pairs M13F and M13R (Invitrogen, Kalsruhe, Germany).

Sequencing and phylogenetic analyses. PCR products were purified with the Quiaquick Spin PCR purification kit (Qiagen). Sequencing was performed by GAG Bioscience (Bremen, Germany) by using the *Taq* Dyedexy Terminator cycle-sequencing kit (Applied Biosystems, Darmstadt, Germany) and an automated sequencer (ABI 377, Perkin Elmer; Lagen, Germany). Retrieved 16S rRNA gene sequences were added to the rRNA gene sequence database of Technical University Munich by using the ARB program package (Strunk et al. 2001). A phylogenetic tree was reconstructed by performing maximum parsimony, neighbor joining and maximum likelihood analyses. Only nearly full-length sequences were used for calculation of the tree. Partial sequences (from DGGE analyses) were inserted into the tree according to parsimony criteria without affecting the overall topology.

The partial sequences of 16S rRNA gene in this study are available from EMBL Genbank under accession numbers AY27445-AY27448 (DGGE bands 1 through 4).

Results

Examination of enrichment cultures. Strictly anoxic marine enrichment cultures from North Sea sediment with iron as electron donor and CO₂ alone or CO₂ and acetate (1 mM) as carbon sources exhibited reduction of sulfate (Dinh et al., 2003) and precipitation of black FeS on the metal and glass wall (Fig. 2A). Free sulfide was not observed. Parallel enrichment cultures with H₂ instead of metallic iron that were included for control also reduced sulfate but remained colorless and formed free sulfide.

For analysis of the enriched populations, 16S rRNA gene fragments retrieved via universal PCR primers were separated by DGGE and sequenced. DGGE profiling and sequence analyses revealed clear differences between the communities enriched with iron and

H₂ (Figs. 3, 4). A prominent DGGE band (no. 1, Fig. 3) that was present in all iron-grown but absent in H₂-grown enrichment cultures affiliated with the *Desulfobacterium* (*Dbm.*) cluster, in particular with strain IS4 isolated from one of the iron cultures (Dinh et al., 2003) and *Dbm. catecholicum*. Another, weaker DGGE band (no. 3, Fig. 3), that was only observed in the acetate-supplemented enrichment culture with iron, affiliated with the *Desulfovibrio* cluster, the closest relatives being strain IS5 from this culture (Dinh et al., 2003) and *Dv. senezii*. Also the enrichment culture with H₂ revealed characteristic bands. One of these (no. 4, Fig. 3), that was not detectable in the iron-grown cultures, was related to *Desulfovibrio aminophilus*. The strongest band from the H₂-grown culture without acetate (no. 2, Fig. 3) affiliated with strain HS2 isolated from this culture (Dinh et al., 2003) and with *Desulfovibrio caledonensis*. A band of the same affiliation was also obtained from the iron-grown enrichment culture without acetate; however, this DGGE band became gradually weaker in further subcultures with iron.

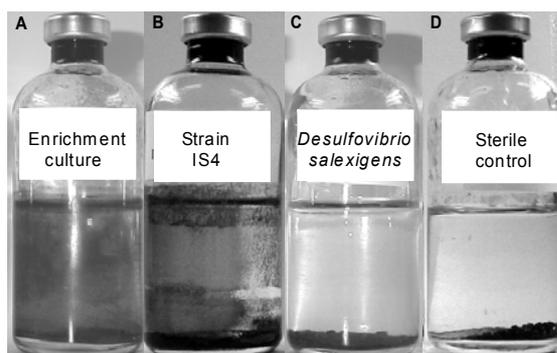


FIG. 2. Cultures of sulfate reducing strains grown in defined medium with iron granules (on the bottom of culture bottles) as the only source of electrons. Growth of the enrichment culture (A) and the novel *Desulfobacterium*-like strain IS4 (B) led to the formation of vast layers of black FeS at the glass wall. In the culture of *Desulfovibrio salexigens* (C), the formed FeS covered merely the iron granules. In the sterile control (D), some FeS was formed on the iron granules by reaction with hydrogen sulfide (1 mM) that was routinely added as reducing agent to all media. The culture of *Desulfovibrio salexigens* was supplemented with acetate as organic carbon source.

A control experiment was also carried out to prove whether the apparently specific enrichment of *Desulfobacterium*-related bacteria was indeed due to metallic iron as a selective electron donor, or to secondary effects of the created chemical environment. Secondary effects that may be envisaged are the strong increase of the pH with iron as electron donor (see next section), and the abundance of ferrous ion, mostly in the form of carbonate (which is more soluble and may be physiologically more effective than FeS). A sample from an enrichment culture with iron was therefore further cultivated without metallic iron at pH 8.5

with added FeCO_3 (5 mmol l^{-1} ; from anoxic equimolar mixture of FeSO_4 and Na_2CO_3) and H_2 as electron donor (with 1 mM acetate as organic carbon source). Molecular analysis revealed disappearance of the *Desulfobacterium* band and appearance of other bands, the most pronounced band affiliating with *Desulfocapsa* (Fig. 3). This suggests that the distinct selection of the *Desulfobacterium*-related bacteria is indeed due to the use of metallic iron as electron donor.

To prove whether the iron-dependent enrichment of the *Desulfobacterium*-related population may be of general relevance or present only a sample-specific phenomenon, an enrichment culture with iron was separately established with sediment from Halong Bay. This enrichment culture yielded the same blackening, DGGE band and 16S rRNA gene sequence (not shown) as the enrichment culture from North Sea sediment.

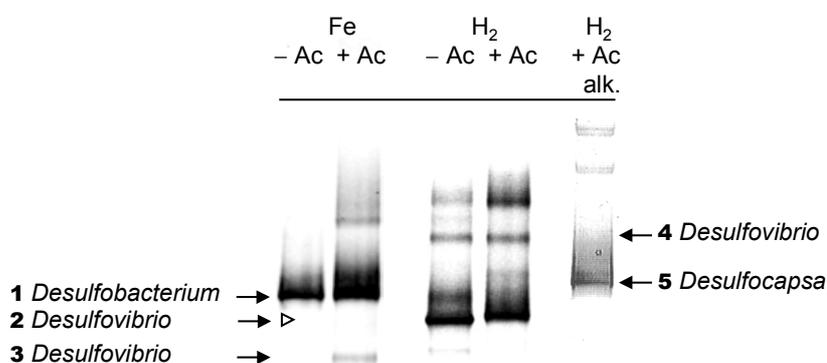


FIG. 3. DGGE profiling of PCR-amplified 16S rRNA gene fragments from sediment-free enrichment cultures (fourth transfer) with metallic iron (Fe) or hydrogen (H_2) as electron donor for sulfate reduction without or with acetate (Ac) as a carbon source in addition to CO_2 . To prove the effect of the chemical conditions around corroding iron without the metal itself, an enrichment culture with H_2 (and acetate) at high pH (8.5; alk.) was established and analyzed for control (right lane). Arrows point at bands that were excised and sequenced (see also Fig. 4). The arrowhead indicates the position of a band that became gradually weaker during subcultivation with iron in the absence of acetate.

Characterization of isolated strains of corrosive sulfate-reducing bacteria. The two sulfate-reducing strains IS4 and IS5, which have been directly isolated with metallic iron from the enrichment culture from North Sea sediment (Dinh et al., 2003), were characterized in more detail. For comparison, strain HS2 from the H_2 -grown enrichment culture and the marine *Desulfovibrio salexigens* were included as "conventional" sulfate-reducing bacteria in some growth experiments. Previously determined relationships on the basis of 16S rRNA gene sequences (Dinh et al., 2003) are depicted together with the affiliations of the presently obtained DGGE bands (Fig. 4).

Strains IS4 and IS5 reduced sulfate with metallic iron at significantly higher rate than representatives of other sulfate-reducing bacteria that have been originally enriched and isolated with H₂, or organic compounds as electron donors; best growth with iron was observed with strain IS4 (Dinh et al., 2003). During growth of strain IS4 with metallic iron, intense deposition of black ferrous sulfide on the iron specimens and glass wall was observed, similar as in the original enrichment culture with iron (Fig. 2A, B). Such vast layers of FeS were not observed in cultures of other SRB with metallic iron (for instance *Desulfovibrio salexigens*; Fig. 2C).

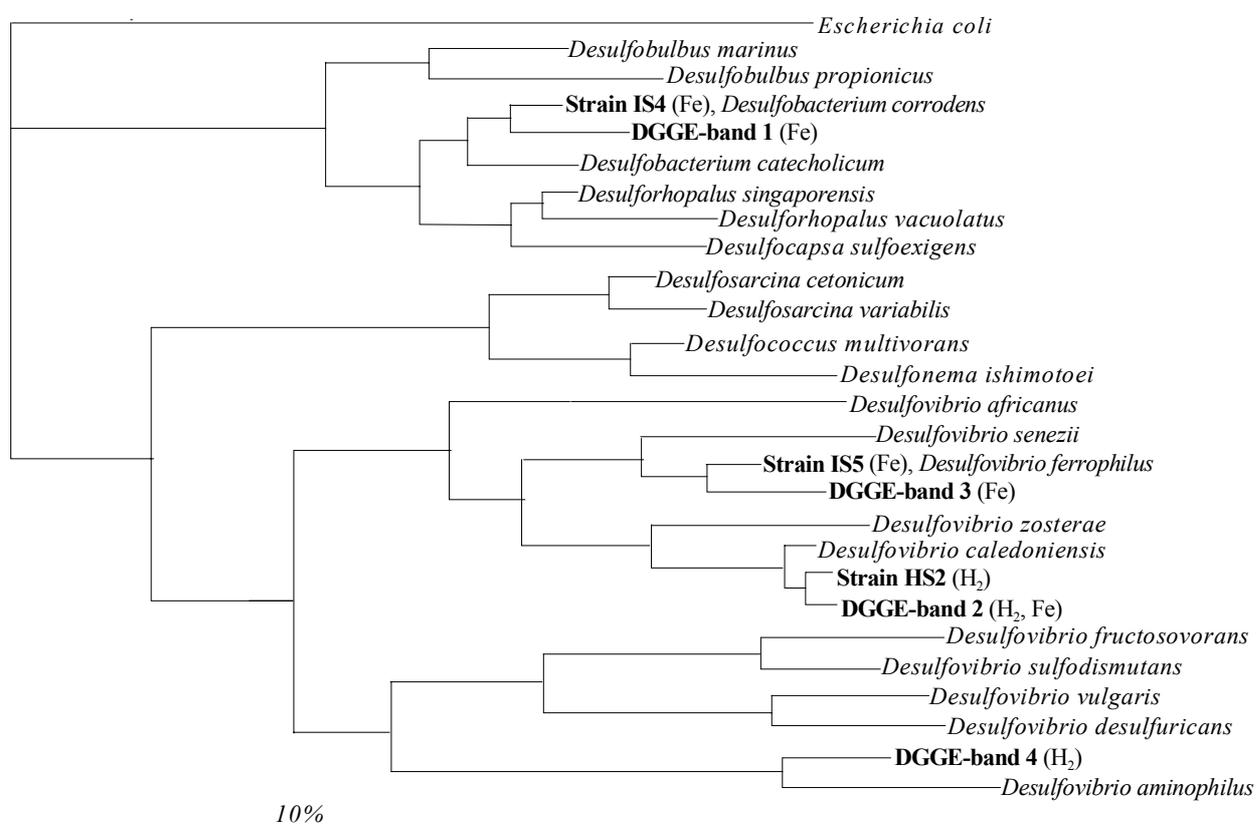


FIG. 4. Phylogenetic affiliation of 16S rRNA gene sequences from sulfate-reducing communities enriched with iron or H₂ (sequences of DGGE bands, Fig. 3), and from isolated pure cultures (sequences from ref [Dinh et al., 2003]). The electron donor used for enrichment or isolation (metallic iron or H₂) is indicated in parentheses. The tree is exclusively based on nearly-full length sequences of pure cultures of Deltaproteobacteria. The 550 bp long sequences from the enrichment cultures were inserted into the tree without affecting the topology. *E. coli* was taken as outgroup. The bar represents 10% sequence divergence.

Strains IS4 and IS5 utilized a limited range of electron donors. Besides iron, only H₂, formate, lactate and pyruvate served as electron donors among various compounds tested. No

growth occurred with ethanol, acetate, propionate, *n*-butyrate, succinate, fumarate, malate, alanine, glutamate, benzoate or catechol. If 20 mM lactate were added and consumed, 18 mM acetate was formed and not consumed further. Considering that sulfate-reducing bacteria assimilate approximately 10% of their organic substrate into cell material (Rabus et al., 2000), this observation is fully in agreement with an incomplete oxidation that ceases at the level of acetate. Among the examined species of sulfate-reducing bacteria, strain IS4 was the fastest with metallic iron but the slowest with H₂ or lactate as electron donors (Fig. 5). Whereas strain IS4 reduced only sulfate, strains IS5 could in addition utilize sulfite or thiosulfate as electron acceptors. In the absence of an inorganic electron acceptor, both strains grew fermentatively with pyruvate. Strain IS4 exhibited in addition poor fermentative growth with lactate. During growth with metallic iron or H₂ and sulfate, strain IS4 could be grown indefinitely in subcultures in the bicarbonate-containing mineral medium without an organic carbon source; still, a slight stimulation by added acetate (1 mM) could be observed during growth with H₂. In contrast, growth of strain IS5 on iron or H₂ depended strictly on acetate as an organic carbon source.

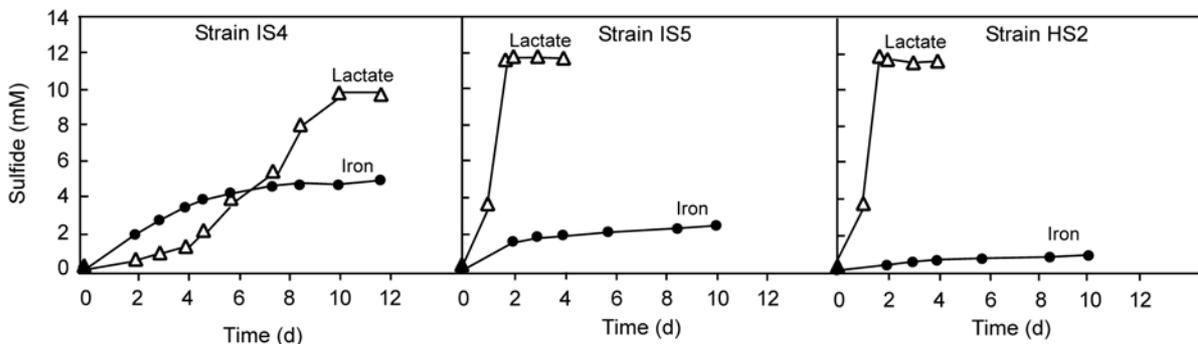


FIG. 5. Sulfate-reduction to sulfide during cultivation of the new isolates of sulfate-reducing bacteria with metallic iron (●) or lactate (Δ). Sulfate reduction with iron as a measure of the corrosive potential exhibits differences between strain *Desulfobacterium corrodens* strain IS4 (highly corrosive), *Desulfovibrio ferrophilus* strain IS5 (moderately corrosive) and a conventionally isolated *Desulfovibrio* species (not directly corrosive, very poor growth with iron). The cultures were supplemented with acetate (1 mM) as organic carbon source. In the lactate-grown cultures, H₂S was measured directly in a colometric assay. In iron-grown cultures, formed H₂S is precipitated as FeS and was therefore determined via microgravimetric sulfate analysis (data adapted from ref. [Dinh et al., 2003]).

During growth of strains IS4 and IS5 on metallic iron, the pH of the medium (originally 7.2) increased above 9 and 8, respectively. In accordance with this, strains IS4 and IS5 exhibited relatively high pH optimum; cell growth and sulfide production from sulfate (both

examined during the initial growth phase were most rapid if the initial pH of the medium was around the above pH values. In accordance with their marine origin, strains IS4 and IS5 required saline medium for growth. With the three media tested (see Materials and Methods), growth in brackish medium was slightly faster than in the full marine medium. No growth occurred in freshwater medium.

Strains IS4 and IS5 also differed with respect to cell attachment. During growth of strain IS4 with iron, cells were microscopically observed mostly in precipitates removed from the iron surface (for scanning electron micrograph see reference Dinh et al.), but only rarely in the free medium. With H₂ or lactate, however, growth occurred in the free medium. However, a tendency to form loose flocs besides freely living cells was usually observed towards the end of growth. In contrast, iron-grown cultures of strain IS5 revealed cell abundance not only in precipitates but also in the free medium. With H₂ or lactate, this strain consistently yielded dense homogeneous growth in the medium.

Discussion

Strains IS4 and IS5 are the first pure cultures of sulfate-reducing bacteria that have been selected and subsequently isolated with metallic iron as the only electron donor for sulfate reduction. A formerly recommended enrichment technique for SRB also employed metallic iron pieces, either as a reductant or for gradual liberation of H₂ in organic media (Butlin and Adams, 1947; Butlin et al., 1949; Schlegel, 1993). Furthermore, a few sulfate-reducing bacteria have been listed (Postgate, 1984) that originate from microenvironments around corroding iron. However, isolation has been always carried out with organic substrates such as lactate and consistently led to *Desulfovibrio* species. A corrosive sulfate-reducing bacterium as represented by the *Desulfobacterium*-related strain IS4 has not been described. Such sulfate-reducing bacteria may have been overlooked for two reasons. First, the common detection of sulfate-reducing bacteria, e.g. during monitoring in sulfidic environments, is based on rapid growth in lactate medium, a property common to *Desulfovibrio* species, whereas strain IS4 exhibits slow growth with lactate (Fig. 5). Second, the presently studied enrichment cultures were established by transfer of iron specimens to subcultures, and strain IS4 was isolated from a homogenized precipitate removed from the iron surface. Common enrichment and isolation techniques usually select for freely dispersed cells.

With metallic iron as the only electron donor, the novel type of sulfate-reducing bacterium, strain IS4, exhibited the most rapid growth among the presently tested species.

Also, the enrichment cultures with metallic iron apparently caused a selection of this species, as shown by DGGE analyses of 16S rRNA gene fragments. On the one hand, the underlying PCR may exhibit biases towards certain sequences and thus not yield a quantitative display of the species diversity. On the other hand, the presently applied PCR and DGGE analysis is commonly regarded as a useful tool to reveal the affiliation of abundant bacteria in environments, and intense DGGE bands usually also reflect dominant organisms (Muyzer et al., 1995; Muyzer et al. 1998). Furthermore, 16S rRNA-targeted whole-cell hybridization with a specific fluorescent oligonucleotide probe revealed that the most abundant cells in precipitates on the corroding iron in the presently studied enrichment culture resembled strain IS4 (Dinh et al., 2003). It may be concluded from these findings and the growth experiments with iron (Fig. 5) (Dinh et al., 2003) that strain IS4 is a typical representative of the sulfate-reducing bacteria that are dominant and mainly responsible for iron corrosion in the enrichment culture. Also the unusually high pH optimum indicates an adaptation to the use of iron as an electron donor. This process causes more alkalization than other reactions of sulfate reduction, which is especially obvious if equations (see introductory part) are reformulated with hydroxyl ions ($4 \text{ Fe} + \text{SO}_4^{2-} + 4 \text{ H}_2\text{O} \rightarrow 3 \text{ Fe}^{2+} + \text{FeS} + 8 \text{ OH}^-$; e.g. vs. $4 \text{ H}_2 + \text{SO}_4^{2-} \rightarrow 3 \text{ H}_2\text{O} + \text{HS}^- + \text{HO}^-$). Such SRB may be also main culprits of iron corrosion in different marine environments. The enrichment of morphologically and phylogenetically similar bacteria from a second study site (Halong Bay) in addition to the original one (North Sea) gives a first hint that the presently characterized type of corrosive sulfate-reducing bacterium is wide-spread. Another hint as to an involvement of *Desulfobacterium*-related sulfate-reducing bacteria in corrosion may be seen in the molecular detection of relatives of the family *Desulfobacteriaceae* in addition to those of the family *Desulfovibrionaceae* in an organic-rich biofilm on corroding iron (Zhang and Fang, 2001). Further studies with enrichment cultures of different origin and samples taken directly from corroding sites (e.g., pipelines) are needed to reveal the significance of *Desulfobacterium*-related sulfate-reducing bacteria such as strain IS4 in the process of anaerobic iron destruction in sulfate-rich environments.

The effective utilization and corrosion of iron as an electron donor by strain IS4 is explained by an assumed direct electron flow from the iron surface into the sulfate-reducing system in the cells (Dinh et al., 2003). A release of significant H_2 concentrations during growth with metallic iron indicated that this compound represents a by-product (Dinh et al., 2003) rather than the direct intermediate postulated by the classical depolarization theory. The release of H_2 can also explain the formation of vast black ferrous sulfide layers in the

enrichment culture and the culture of strain IS4. Surface-attached cells of this bacterium not only reduce sulfate to sulfide that is immediately precipitated as FeS on the iron; through constitutive hydrogenase, the attached cells also release excess reducing equivalents as H₂ that is subsequently used by other cells of strain IS4 or *Desulfovibrio* (in the enrichment culture) in the free medium and on the glass wall for the reduction of sulfate to form additional ferrous sulfide throughout the bottle (Fig. 6).

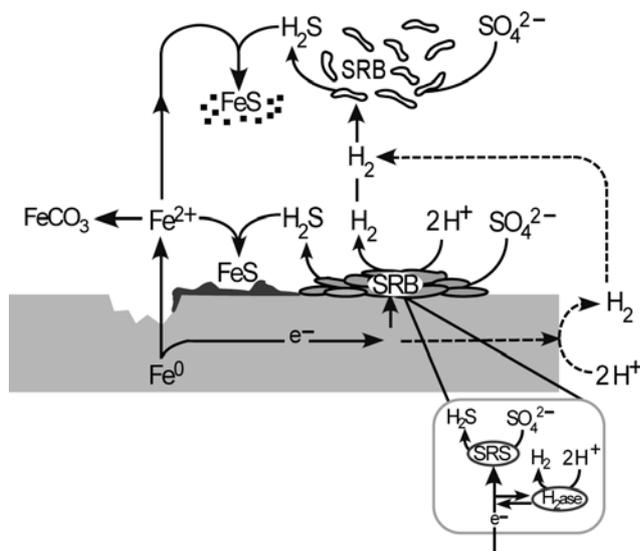


FIG. 6. Proposed simplified model of anaerobic corrosion mediated by sulfate-reducing bacteria in the enrichment culture and under *in situ* conditions. Corrosive species of SRB, especially such as the newly isolated *Desulfobacterium corrodens* strain IS4, gain electrons for sulfate reduction in direct contact with the iron (further discussion in ref. [Dinh et al., 2003]). Excess electrons from an imbalanced electron flow lead to formation of H₂ through constitutive hydrogenase (H₂ase) on a "side-path". This and additional H₂ formed by slow chemical reaction in the aqueous medium is utilized by sulfate-reducing bacteria not living in direct contact with the iron. Hence, produced sulfide precipitates Fe²⁺ ions as FeS not only directly on the iron surface but also in other parts of the aqueous surrounding (such as the glass wall in Fig. 2A, B). For convenience, stoichiometric factors are not included. The stoichiometrically correct equation in bicarbonate-containing water is $4 \text{ Fe} + \text{SO}_4^{2-} + 3 \text{ HCO}_3^- + 5 \text{ H}^+ \rightarrow \text{FeS} + 3 \text{ FeCO}_3 + 4 \text{ H}_2\text{O}$ (for stoichiometry without bicarbonate see introductory section).

Taxonomic aspects, and description of *Desulfobacterium corrodens* and *Desulfovibrio ferrophilus*. The physiologically most striking property of strain IS4 is the ability to utilize iron as electron donor for sulfate reduction. With respect to organotrophic growth, strain IS4 is restricted to few substrates only and thus differs from many other members of the *Desulfobacterium* cluster which are often nutritionally versatile. Also the lack of the capacity for complete oxidation in strain IS4 is untypical for members of this cluster. On the other hand, strain IS4 can grow autotrophically, a property common to many *Desulfobacterium*

species (Brysch et al., 1987; Widdel and Bak, 1992). Based on the distinct physiological properties of strain IS4 and its phylogenetic distance to *Desulfobacterium catecholicum* as the closest cultivated relative (16S rRNA sequence similarity, 95%), strain IS4 is regarded as a new species of the genus *Desulfobacterium*, and the name *Desulfobacterium corrodens* is proposed. The other isolate from the enrichment culture, strain IS5, is apparently less corrosive toward iron than strain IS4, but more corrosive than the other sulfate-reducing bacteria tested. Otherwise, this isolate resembles nutritionally, morphologically and phylogenetically common *Desulfovibrio* species. However, due to the significant phylogenetic distance to its closest cultivated relative, *Desulfovibrio senezii* (16S rRNA gene sequence similarity, 92%), strain IS5 is also regarded as a new species of the genus; the name *Desulfovibrio ferrophilus* is proposed.

Desulfobacterium corrodens. *cor.ro'dens* L. part. *corrodens* of L. tr. v. *corrodo* gnaw, corrode. Cells are 0.8–1 by 4–8 μm in size. Prolonged cells, formation of long chains and growth in flocs are usually observed toward the end of growth. Motility has not been observed. Brackish to marine salt concentrations are required for growth. The pH optimum is around 9; the temperature optimum is at 28–30 °C. Metallic iron, H₂, formate, lactate, pyruvate serve as electron donors for sulfate reduction. Fermentative growth occurs readily with pyruvate and poorly with lactate. Sulfate is the only electron acceptor utilized. Autotrophic growth is possible. The G+C content of the DNA is 51.9 mol% (determined by HPLC analysis). The type strain, IS4, has been isolated from from anoxic marine sediment (near Wilhemshaven, North Sea) and has been deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ) under number 15630.

Desulfovibrio ferrophilus. *fer.ro'phi.lus* comp. M. L. adj. *ferrophilus* from L. n. *ferrum* iron, and Gr. v. *philo* like, *ferrophilus* that likes iron. Curved motile cells (vibrioids), 0.8–1 by 4–8 μm in size. Metallic iron, H₂, formate, lactate, pyruvate serve as electron donors for sulfate reduction. Sulfate, sulfite and thiosulfate are used as terminal electron acceptors. Fermentative growth is observed with pyruvate. Brackish to marine salt concentrations are required for growth. The pH optimum is around 8; the temperature optimum is 28–30 °C. The G+C content of the DNA is 55.8 mol% (determined by HPLC analysis). The type strain, IS5, has been isolated from anoxic marine sediment (near Wilhemshaven, North Sea) and has been deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ) under number 15579.

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