Baltic Sea methanogens compete with acetogens for electrons from metallic iron

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Abstract
Microbially induced corrosion of metallic iron (Fe0)-containing structures is an environmental and economic hazard. Methanogens are abundant in low-sulfide environments and yet their specific role in Fe0 corrosion is poorly understood. In this study, Sporomusa and Methanosarcina dominated enrichments from Baltic Sea methanogenic sediments that were established with Fe0 as the sole electron donor and CO2 as the electron acceptor. The Baltic-Sporomusa was phylogenetically affiliated to the electroactive acetogen S. silvacetica. Baltic-Sporomusa adjusted rapidly to growth on H2, spent filtrate enhanced growth of this acetogen suggesting that it was using endogenous enzymes to retrieve electrons and produce acetate. Previous studies have proposed that acetate produced by acetogens can feed commensal acetoclastic methanogens such as Methanosarcina. However, Baltic-methanogens could not generate methane from acetate, plus the decrease or absence of acetogens stimulated their growth. The decrease in numbers of Sporomusa was concurrent with an upsurge in Methanosarcina and increased methane production, suggesting that methanogens compete with acetogens for electrons from Fe0. Furthermore, Baltic-methanogens were unable to use H2 (1.5 atm) for methanogenesis and were inhibited by spent filtrate additions, indicating that enzymatically produced H2 is not a favorable electron donor. We hypothesize that Baltic-methanogens retrieve electrons from Fe0 via a yet enigmatic direct electron uptake mechanism.

Introduction
Microbially induced corrosion (MIC) accounts for 20% of the total corrosion costs for the oil and gas industries [1, 2]. Additionally, chemical leaks from corroded waste containers cause health and environmental problems [3, 4]. Previous studies have primarily focused on MIC in sulfide-rich marine environments, where sulfate-reducing bacteria are predominantly causing corrosion [5]. However, in low-sulfide environments such as the Bothnian Bay, Baltic Sea [6], cooperative interactions between microorganisms (i.e. methanogens and acetogens) may be responsible for corrosion of infrastructure [7–10]. Microbial cooperation was proposed to enhance corrosion rates [11], but it is poorly understood. Of the methanogens, particularly Methanosarcina have been suggested to play an essential role in corrosion. Methanosarcina are frequently associated with corrosive Methanococcus and Methanobacterium strains described to date (Methanococcus maripaludis strains KA1, Mic1c10, MM1264, and Methanobacterium strain IM1 [13–16]), none belongs to the genus Methanosarcina. Studies of corrosive Methanococcus and Methanobacterium have shown that H2 generated abiotically by Fe0 cannot provide sufficient electrons to account for all the methane being produced by these species during growth in the

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presence of Fe\textsuperscript{0} [13–17]. Consequently, the electron uptake mechanisms proposed for these different methanogenic strains included (1) a direct uptake route [15, 17] or (2) an extracellular enzyme-mediated electron uptake route [18, 19].

(1) Direct electron uptake from Fe\textsuperscript{0} by Methanobacterium strain IM1 was suggested as an alternative to abiotic-H\textsubscript{2} uptake because this strain generated more methane (CH\textsubscript{4}) from Fe\textsuperscript{0} oxidation than a H\textsubscript{2}-utilizing M. maripaludis strain [15] with low H\textsubscript{2}-uptake thresholds [20]. IM1 also produced methane when a cathode poised at a potential unfavorable for abiotic H\textsubscript{2} evolution was the sole source of electrons [17]. However, the mechanism utilized by IM1 for electron uptake directly from Fe\textsuperscript{0} or electrodes is unknown. Also, it is unknown whether other methanogens have similar capabilities.

Methanosarcina species were previously shown to carry out Fe\textsuperscript{0}-dependent methanogenesis presumably by using abiotic-H\textsubscript{2} evolved at the Fe\textsuperscript{0}-surface [21]. However, some Methanosarcina cannot use H\textsubscript{2} [22–26], and even those Methanosarcina species that are capable of hydrogenotrophic methanogenesis have high thresholds for H\textsubscript{2}-uptake [27, 28]. Thus, Methanosarcina species would not be competitive at abiotic-H\textsubscript{2} removal from the Fe\textsuperscript{0} surface. Nonetheless, Methanosarcina-species dominate on corroded Fe\textsuperscript{0}-structures [7–10]. Therefore, we propose that Methanosarcina retrieves electrons directly from Fe\textsuperscript{0}, extrapolating from recent findings that Methanosarcina can retrieve extracellular electrons directly from poised electrodes [29, 30], electrogenic syntrophic partners [31, 32], or electrically conductive particles [31–34]. Plausible scenarios for direct electron uptake in Methanosarcina have only recently been substantiated, using comparative transcriptomics [35].

(2) In addition to direct extracellular electron uptake, a second strategy making use of extracellular enzymes to capture electrons from Fe\textsuperscript{0} was described in methanogens [16, 18, 19]. Enzymes like hydrogenases, formate dehydrogenases, or the heterodisulfide reductase supercomplex produced by methanogens can generate H\textsubscript{2} or formate from Fe\textsuperscript{0}-derived electrons [16, 18, 19]. It is not clear whether an extracellular enzyme-dependent strategy would be competitive in corrosive environments. Moribund cells may release enzymes like hydrogenases into their extracellular milieu [19] that can capture electrons freed during Fe\textsuperscript{0}-oxidation to reduce protons from solution to H\textsubscript{2} [36, 37]. H\textsubscript{2} could then be used non-specifically by a variety of H\textsubscript{2}-utilizers. Sensitive anaerobic enzymes tend to only be stable for a few days outside of the cell [38]. However, Fe\textsuperscript{2+} released during the corrosion process may further stabilize these enzymes [39]. Hydrogenase-mediated H\textsubscript{2} production at the Fe\textsuperscript{0} surface appears to generate sufficient H\textsubscript{2} for the growth of hydrogenotrophic methanogens [16, 19].

Corroded infrastructure often harbors both Methanosarcina-methanogens and acetogens, where Methanosarcina is thought to play a role in Fe\textsuperscript{0}-corrosion [7–10]. However, Methanosarcina\textsuperscript{*}s role was assumed to be indirect and dependent on cooperation with other corrosive organisms. For example, Methanosarcina was proposed to utilize acetate produced by acetogens actively corroding Fe\textsuperscript{0}. In this study, we investigated the theory that acetoclastic methanogens like Methanosarcina require cooperative interactions with acetogens to corrode Fe\textsuperscript{0}. We used Fe\textsuperscript{0} to enrich for Methanosarcina species from sediments collected off the coast of Bothnia. Molecular and physiological tests were used to investigate the role of methanogens and their possible synergy with co-occurring microbes during Fe\textsuperscript{0} corrosion. We present evidence that Baltic-methanogens perform Fe\textsuperscript{0}-dependent methanogenesis and compete with acetogens for access to Fe\textsuperscript{0}. Specific inhibition experiments indicate that two different mechanisms for Fe\textsuperscript{0}-dependent electron uptake by Baltic-acetogens and methanogens are feasible.

Materials and methods

Baltic-Sea-enrichment cultures

We collected sediment cores from the Bothnian Bay, Baltic Sea at a water depth of 15 m (65°43.6′N and 22°26.8′E; station RA2) during August 2014 [40]. The sediment had a temperature of 15°C and an in situ salinity of 0.5. The mineral content was low in insoluble manganese oxides, high in insoluble FeS, and high in crystalline iron oxides, such as semiconductive goethite or conductive magnetite, as previously described [40].

Enrichment cultures were prepared using sediment from the methanogenic zone (30–36 cm) under aseptic and anoxic conditions as previously described [40], but with the addition of 100 g/L iron granules, and exclusion of sulfide as a reducing agent, which was instead replaced with an additional 2 mM cysteine (c\textsubscript{f} = 3 mM). We prepared all subsequent transfers in 50 mL blue chlorobutyl-rubber-stoppered glass vials with an anoxic headspace of CO\textsubscript{2}/N\textsubscript{2} (20:80, v/v). For all enrichment incubations, we used a DSM120-modified medium (modifications: 0.6 g/L NaCl, without casitone, sodium acetate, methanol, or Na\textsubscript{2}S\times9H\textsubscript{2}O). For Fe\textsuperscript{0} incubations, we added to the media as sole electron donor iron granules (99.98%, ThermoFisher, Germany) or iron coupons (3 cm x 1 cm x 1 mm). Other electron donors tested included H\textsubscript{2} (ca. 1.5 atm) and acetate (10 mM).

All culture experiments were carried out in at least triplicate and sometimes up to 10 replicates. As soon as methane production reached stationary phase, we transferred cells into fresh media with Fe\textsuperscript{0}. Cultures were shaken...
vigorously to remove cells from Fe\textsuperscript{0}-granules. We then used 10–20\% of the dispersed cells to inoculated fresh Fe\textsuperscript{0}-media for subsequent transfers. The temperature used for all incubations was 20 °C. To reach stationary, initial enrichments required circa 6 months, whereas later transfers (T3) took circa 3 months and most recent incubations (T10) took only 1–2 months.

Abiotic control experiments lacked cells. However, we used the same Fe\textsuperscript{0}-media and incubation conditions as done for experiments with cells.

Inhibition experiments were carried out by the addition of inhibitors specific for methanogens or bacteria. A methanogenesis inhibitor (2 mM \textit{N}-bromoethane sulfonate [41]) was added to the culture media to generate a bacteria-only culture. In order to generate a methanogen-only culture, we added a mixture of antibiotics (200 µg/mL kanamycin and 100 µg/mL ampicillin) to the culture media. Experiments with inhibitors were run parallel to controls lacking inhibitors.

Spent filtrate addition experiments were carried out by the addition of 1 mL spent media from a stationary culture to a new culture as described previously [40]. The spent media of a \textit{Sporomusa} acetogen and a \textit{Methanococcus} methanogen were previously shown to contain electroactive enzymes, which retrieve electrons from Fe\textsuperscript{0} or electrodes for proton reduction to H\textsubscript{2} [42].

Downstream analyses, DNA extractions, substrate evaluations, and microscopy were performed after the fifth consecutive transfer on Fe\textsuperscript{0}.

**Chemical analyses**

To determine methane and H\textsubscript{2} concentrations, we used a Trace 1300 gas chromatography system (Thermo Scientific, Italy) equipped with a thermal conductivity detector (TCD), an injector operated at 150 °C and a detector at 200 °C with 1.0 mL/min reference gas flow. The oven temperature was constant at 70 °C. A TG-BOND Msieve 5A column (Thermo Scientific; 30-m length, 0.53-mm i.d., and 20-µm film thickness) was used with argon as the carrier gas with a set flow at 25 mL/min. The GC was controlled and automated with the Chromeleon software ( Dionex, Version 7). Using this setup, the minimum detection limit for methane and H\textsubscript{2} was 5 µM.

For determination of acetate concentrations, we used a Dionex ICS-1500 Ion Chromatography System (ICS-1500) equipped with the AS50 autosampler, and an IonPac AS22 column coupled to a conductivity detector (31 mA). For separation of volatile fatty acids, we used 4.5 mM Na\textsubscript{2}CO\textsubscript{3} with 1.4 mM NaHCO\textsubscript{3} as eluent. The run was isothermal at 30 °C with a flow rate of 1.2 mL/min.

For determination of ferrous iron (Fe\textsuperscript{2+}) produced by Fe\textsuperscript{0}-oxidation in our cultures, we dissolved Fe\textsuperscript{2+} in 0.67 M HCl (containing 0.67 M hexamethylenetetramine to avoid dissolution of metallic iron) and quantified Fe\textsuperscript{2+} concentrations colorimetrically with the ferrozine assay [43].

For elemental analyses of the gray-black crust that formed on Fe\textsuperscript{0}-coupons after 2 months when cells were present, the crust was scraped off the Fe\textsuperscript{0}-coupons and dried in an anoxic glove box. Mass spectrometry informed on the content of carbonate and organic carbon. For total reduced inorganic sulfur determination (including iron monosulfides, pyrite, and S\textsuperscript{0}) we performed hot chromium distillation [44]. The organic carbon quantification took place after acidification with HCl. We calculated the value for the carbon in carbonates by subtracting the organic-carbon values remaining after acidification from the total unacidified carbon (C-total).

**DNA purification from microbial enrichments**

DNA purification was performed using a combination of two commercially available kits; the MasterPure™ Complete DNA and RNA Purification Kit (Epicenter, Madison, Wi, USA), and the Fast Prep spin MP™ kit for soil (Mobio/Qiagen, Hildesheim, Germany). For DNA extraction, we pelleted 10 mL cells, either by harvesting an entire culture grown on Fe\textsuperscript{0} or by removing 10 mL from a larger volume after vigorous shaking the Fe\textsuperscript{0}-cultures in order to detach cells from the Fe\textsuperscript{0}-surface. We used an Epicenter kit to initiate the DNA extraction with the following modifications to the manufacturer’s protocol: a three-fold higher concentration of proteinase K was added to ensure cell lysis, and a prolonged incubation time at 65 °C was performed until the color of the samples changed from black to brown (the brown pellet gave higher DNA extraction efficiencies). After DNA extraction, we used the Fast Prep spin MP™ kit for soil to carry out RNase treatment and protein precipitation. An advantage of this kit is that it allowed removal of the high iron content, while simultaneously purifying DNA on a binding matrix. Quality and quantity of genomic DNA were determined by electrophoresis on a 1% agarose gel and by UV spectrophotometry on a mySPEC spectrophotometer (VWR®, Germany).

**Metagenome analyses**

After a single whole-genome amplification cycle, random shotgun metagenome sequencing was performed commercially (Macrogen/Europe) using the Illumina HiSeq2500 platform. We merged the unassembled DNA sequences, checked for quality, and annotated using the Metagenomics Rapid Annotation (MG-RAST) server (vs. 4.03) with default parameters [45]. Shotgun metagenome sequencing resulted in 10,739 high-quality assembled reads of a total of 10,749 with an average length of 167 bp. We obtained metagenome taxonomy information using the databases
available in MG-RAST, including Silva [46], RDP [47], Greengenes [48], and RefSeq [49]. For the metagenome taxonomy, the horizontal asymptote of the rarefaction curve indicated complete coverage of the prokaryotic diversity in these samples. For metagenome taxonomy analyses, we used the default MG-RAST cutoff parameters: e-value of 1E–5, a minimum identity of 60%, and a maximum alignment length of 15 bp. The metagenome data is available at MG-RAST with this ID: MGM4796413.3.

16S rRNA gene sequence analyses

General Archaeal and Bacterial primers (Table 1SM) were used to perform PCR amplification of the 16S rRNA gene from the isolated DNA. PCR reactions contained in a final volume of 50 µL, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of each primer, and 1U Promega Taq polymerase, and 10x PCR reaction buffer. PCR reactions included an initial denaturation step at 94 °C for 10 min; then 35 cycles of denaturation at 94 °C for 30 s, annealing at the specific annealing temperature for the primer pair (Table 1SM) for 30 s, and extension at 72 °C for 90 s; and a final extension cycle at 72 °C for 10 min. Next, we cloned PCR products with the TOPO® TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). PCR products from individual clones were amplified with M13-vector primers and sent to the Institute of Clinical Molecular Biology in Kiel for Sanger sequencing. Sequences were analyzed using the Geneious® software package, version 11.0.4 [50], and compared against the NCBI GenBank DNA database using BLAST. Consensus sequences for Archaea and Bacteria (97% identity) were assembled using ClustalW implemented within Geneious. Consensus 16S rRNA gene sequences were used to construct maximum likelihood phylogenetic trees in Geneious using RaxML [51]. We deposited sequences in GenBank, under the accession number: MK433201.

Quantitative PCR

Extracted DNA was used for 16S rRNA gene quantification via qPCR with specific Sporomusa, Methanosarcina, and general Bacteria primers (Table 1SM). For quantification of the members within the corrosive Baltic Sea community, we carried out qPCR assays on duplicate cultures harvested at different times during their growth; 18 days (T10) and 60 days (T9). For each biological replicate, we run quadruplicate qPCR reactions alongside quadruplicate standards (10^1–10^8 16S rRNA gene copies per ml). All standards were prepared as previously described [40, 52].

We prepared the qPCR reaction mix as described before in a final volume of 25 µl of which 10 µl were a 5Prime Hot Master Mix, 0.25 µl BSA (stock 10 mg/ml), 1 µl of forward and reverse primer (10 µM stock each) and 1 µl of a template [40]. The qPCR amplification ran as follows: 2 min hot start at 94 °C, 1 min denaturation at 94 °C, 1 min at the annealing temperature appropriate for the primer pair used (Table 1SM) and 2 min extension at 72 °C. Steps two to four (denaturation, annealing, and extension) were repeated 40 times. The final step was a 10 min elongation step at 72 °C and storage at 4 °C.

Fluorescence in situ hybridization

To fix cells, we added 2% microscopy grade paraformaldehyde (PFA, 16%) directly to anaerobic cultures and incubated them for 2 h at room temperature. Then all cells were collected with the Fe0-granules via centrifugation at 10,000 rpm for 10 min. We gently sonicated (20% intensity; 5 × 5 s) to detach cells from the Fe0-granules. Then we collected 50 µL of the resuspended cells by filtration on 0.22 µm filters. We hybridized cells with specific probes (final concentration 5 ng/µL) for Methanosarcina (MX821) using the formamide concentration specified in Table 1SM, followed by 2 h hybridization at 46 °C and 15 min. washing at 48 °C [53]. For counterstaining, we incubated the air-dried filters for 3–5 min with a DNA fluorescent stain: 4′,6-diamidino-2-phenylindole (DAPI; 1 µg/ml).

Epifluorescence microscopy

To confirm the presence or absence of methanogens, we also used their natural autofluorescence due to coenzyme F420 and visualized the cells on an epifluorescence microscope equipped with a 420 nm excitation filter as previously described [54]. To visualize cells, we used an upright epifluorescence microscope from Zeiss (Axioskop A1) equipped with a Cy3 (excitation 549 nm, emission 562 nm), a DAPI (excitation 359 nm, emission 461 nm), and an F420 filter set (excitation 420 nm, emission 480 nm). For image acquisition, we used a digital CCD camera (AxioCam) controlled by an Axiovision vs. 4.7 software.

Scanning electron microscopy and sample preparation

We carried out SEM visualization on cells from the fifth transfer that have been growing on Fe3+-coupons for circa 3 months. We removed the excess culture media and directly fixed cells attached to the Fe0-coupon in the anaerobic culture vials using a mix of 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Cells were incubated at 4 °C for 12 h, washed in phosphate buffer, dehydrated with anoxic ethanol at increasing concentrations (35%, 50%, 70%, 80%, 90%, 95%, 100%, and three times in 100% v/v; each step for 10 min). The Fe0-coupons were then
chemically dried with hexamethyldisilazane for 30 min [55] and traces of hexamethyldisilazane were evaporated under N2. We stored dried-out Fe0-coupons in the culture bottle under N2-gas before electron microscopy. Scanning electron microscopy (SEM) was performed with a FESEM Magellan 400 at 5.0 kV at the microscopy facility of the University of Massachusetts, Amherst, USA.

Results and discussion

Previous studies of corrosive non-sulfidic environments attributed corrosion to syntrophic interactions between acetogens (e.g. Clostridium) and acetate-utilizing methanogens (Methanosarcinales) [7–10]. Here we challenge this assumption and demonstrate that acetogens and methanogens compete for electrons from Fe0, rather than operating cooperatively.

A corrosive community became enriched from methanogenic sediment collected off the Swedish coast using Fe0 as the sole electron donor and CO2 as the sole electron acceptor. After the establishment of original slurries on Fe0 (25% sediment), cultures were transferred sequentially into fresh Fe0-containing media using a 10–20% inoculum for the next 3 years.

Original slurries from Baltic Sea sediments provided with Fe0 generated circa five times more methane (Fig. 1a) and four times more acetate (Fig. 1a) than parallel incubations without Fe0 (Fig. 1b). Surprisingly, these Fe0-containing slurries accumulated acetate (Fig. 1), which was not consumed by the Methanosarcina known to harbor these sediments [40].

After three transfers, incubations became sediment-free as determined by (i) visual inspection, (ii) microscopy, and (iii) the fact that the inoculum did not lead to product formation from carryover of electron donors in incubations without external electron donors (Figs. 1c and 1SM). To verify for acetate utilization by acetoclastic methanogens, Fe0 was replaced with 10 mM acetate as the sole substrate for growth. Acetate did not lead to methane production after 2 months of incubation, whereas Fe0 did (Fig. 1c), indicating that enriched Baltic-methanogens became adjusted to Fe0-dependent methanogenesis, and were not capable of acetoclastic methanogenesis during the given time frame of 2 months.

These sediment-free cultures formed a black crust on the surface of the metal, which was absent in abiotic incubations (Fig. 1c—inset). Under similar conditions (non-sulfidic, carbonate-buffered, pH~7), Fe0 is oxidized primarily into Fe2+-carbonates such as siderite [15, 56, 57]. Microorganisms like methanogens or acetogens accelerate Fe0-oxidation to Fe2+ via processes that are energetically more favorable than the abiotic reaction (Table 1). During the eighth transfer, we removed and analyzed the gray-black precipitate formed on Fe0 by the Baltic Sea methanogenic community. We determined that the precipitate had a high carbonate content (ca. 50% by weight in FeCO3 equivalents), but low reduced inorganic sulfur content (ca. 0.1%; including sulfides), consistent with the formation of iron carbonates, like siderite. The remaining organic carbon content was ~1%.

Fig. 1 Initial slurrys established from methanogenic sediments collected off the coast of the Baltic Sea and third transfer incubations with Fe0 or acetate as electron donors vs. parallel control incubations without electron donors. a Slurries incubated with Fe0 or b without Fe0 (n = 3). Electron conversions into products (methane and acetate) are presented as mM electron equivalents (mM eeq) taking into account that a mol methane/acetate requires 8 mols electrons according to the reactions: CO2 + 8e− + 8H+ → CH4 + 2H2O (methanogenesis) and 2 CO2 + 8e− + 8H+ → CH3COOH + 2H2O (acetogenesis). c Fe0-dependent methane production in the third successive passage (sediment-free). However, methane was undetected in electron-donor free controls and when 10 mM acetate replaced Fe0 as sole electron donor. The electron donor-free control was used to probe for sediment carryover substrates (c—inset). A gray-black corrosion product was observed only in the presence of cells and not in their absence (c—inset).
Enhanced Fe⁰-oxidation to Fe²⁺ by Baltic Sea acetogens and methanogens

Fe⁰ corrosion was assessed using ferrous iron (Fe²⁺) accumulation as a proxy for corrosion (Fig. 2a, b), as done before, particularly in studies documenting corrosion by methanogens [13–15, 58, 59].

In our incubations, the corrosive microbial community started Fe⁰-oxidation to Fe²⁺ immediately and persisted for circa 25 days (Fig. 2a). The presence of cells led to a tripling of the Fe²⁺ yield per day (44.7 ± 4.6 µM/day) compared to the background Fe²⁺ yield observed in abiotic controls (16.9 ± 10 µM/day) (Fig. 2b). The daily increase of Fe²⁺ in the presence of a Baltic-community indicates that the community was more corrosive than abiotic controls.

Fe⁰-corrosion under non-sulfidic, carbonate-rich conditions in the absence of electron acceptors other than CO₂ can be attributed to two possible metabolisms: Fe⁰-dependent methanogenesis or Fe⁰-dependent acetogenesis. Theoretically, Fe⁰-dependent methanogenesis is energetically more favorable than Fe⁰-dependent acetogenesis, but both are energetically more favorable than abiotic Fe⁰-dissolution (Table 1). Therefore, to better understand the interplay between acetogens and methanogens within the corrosive microbial community we (1) monitored product evolution and (2) inhibited various metabolic groups in order to determine the corrosive potential of each surviving group.

Electron recoveries from Fe⁰

After eight transfers on Fe⁰-containing medium, the Baltic Sea corrosive enrichment cultures exhibited a quick Fe⁰-dependent acetogenic phase (days 5–10) followed by a slow methanogenic phase (days 10–25) (Fig. 2d). During the acetogenic phase, acetogens were able to convert 2.2 ± 0.1 mM/day electron equivalents from Fe⁰ to form acetate (Fig. 2d). As soon as the acetogenic phase stopped, the methanogenic-phase began, and methanogens recuperated 0.2 ± 0.05 mM electron equivalents from Fe⁰ into methane daily. After 25 days, the community routed more electrons into acetate than into methane (Fig. 2d). Thus, it appears that acetogens outcompeted methanogens for access to electrons from Fe⁰.

Competition between acetogens and methanogens

We evaluated whether methanogens were in competition with acetogens for electrons from Fe⁰ by testing whether methanogens functioned better without acetogens. In order to test this, we inhibited acetogens and other bacteria using antibiotic additions (kanamycin and ampicillin). With acetogens inhibited, methanogens oxidized Fe⁰ to Fe²⁺ at rates above abiotic controls (Fig. 2e) and similar to those observed for the entire community (Fig. 2d). After 15 days, methanogens alone produced six-times more methane (3.8 ± 0.7 mM electron equivalents/eeq CH₄) (Fig. 2e) than they did when they were co-existing with bacteria in a mixed community (0.6 ± 0.2 mM eq CH₄) (Fig. 2d). During the first 5 days of the methanogenic-phase (days 10–15), electron recoveries into methane were higher (0.81 ± 0.06 mM eeq CH₄) than expected from the rates achievable if cells were dependent on the production of abiotic H₂ production (0.65 ± 0.09 mM eeq H₂). Nevertheless, electron recoveries decreased by half (0.42 ± 0.1 mM eeq CH₄) (Fig. 2e), possibly due to competition for Fe⁰ with acetogens that developed antibiotic resistance and generated 2.5 mM eeq acetate. However, even brief inhibition periods of the acetogens led to significantly higher methanogenic activity (six-fold), indicating that acetogens inhibited methanogenesis on Fe⁰. Sub-optimal methane production suggests that methanogens may experience decreased access to electrons from Fe⁰ due to the competitive exclusion by acetogens.

To test whether methanogens impacted the growth of acetogens, we inhibited methanogens with BES, a methyl–CoA analog [41]. In the presence of the methanogenic inhibitor, methanogens were rendered inactive throughout the incubation (Fig. 2f). Nevertheless, acetogens alone were able to oxidize Fe⁰ to Fe²⁺ (Fig. 2a, b), while producing more acetate (14%; p = 0.0001) than they did within the mixed community (Fig. 2f). These data suggest that methanogens constrain the growth of acetogens.

Table 1 Possible reactions occurring at the Fe⁰ surface in non-sulfidic carbonate-buffered media

| Process | Reaction | Delta G⁰ |
|---------|----------|----------|
| Abiotic Fe⁰ dissolution in carbonate-buffered systems | Fe⁰ + HCO₃⁻ + H⁺ → FeCO₃ + 2H⁺ + 2e⁻ | −79.9 kJ/mol Fe⁰-used |
| Abiotic H₂—evolution | 2H⁺ + 2e⁻ → H₂ | |
| Methanogenesis from Fe⁰ | 4Fe⁰ + CO₂ + 4HCO₃⁻ + 4H⁺ → CH₄ + 4FeCO₃ + 2H₂O | −111.5 kJ/mol Fe⁰-used |
| Acetogenesis from Fe⁰ | 4Fe⁰ + 2CO₂ + 4HCO₃⁻ + 4H⁺ → CH₃COOH + 4FeCO₃ + 2H₂O | −97 kJ/mol Fe⁰-used |
These results indicate that acetogens and methanogens negatively affect one another when competing for Fe$^0$ as the sole electron donor (Fig. 3).

**Contrasting mechanisms of electron uptake from Fe$^0$ in Baltic-acetogens and methanogens**

To investigate the possible mechanisms of electron uptake by Baltic-acetogens and methanogens, we compared electron recoveries in abiotic controls vs. those in the presence of cells. In the absence of cells, Fe$^0$ released 0.65 ± 0.09 mM/day electron equivalents as H$_2$ (Fig. 2c) continuously for 25 days. When acetogens were present, electron equivalents were recovered 3.5 times faster than expected from abiotic H$_2$ (Fig. 2f), excluding electron recovery into biomass unaccounted for during the experiments. Thus, acetogens likely used an alternative mechanism to access electrons from Fe$^0$ easily and accelerate acetogenesis.

Acetogens have been shown to use two different mechanisms for electron uptake from Fe$^0$ facilitated by enzymes evolving H$_2$ [16, 19] or by direct-electron uptake [60–62]. The latter is plausible because several acetogens can grow on electrodes poised at potentials that do not generate abiotic H$_2$ [60–62]. On the other hand, previous studies illustrated efficient enzymatic-mediated electron uptake by methanogens for H$_2$ and CH$_4$.
uptake from Fe\(^0\) using a purified *Clostridium* [FeFe]-hydrogenase, which retrieves electrons directly from Fe\(^0\) for proton oxidation to H\(_2\) [36, 37]. Unlike [NiFe]-hydrogenases from methanogens, the [FeFe]-hydrogenases of *Clostridium* are effective at oxidizing H\(_+\) [63] and quickly evolving H\(_2\) that could serve as an electron donor for Baltic-acetogens. Therefore, we had to determine whether Baltic-acetogens (i) utilize H\(_2\), (ii) are stimulated by endogenous enzymes, or (iii) use an alternative direct electron uptake mechanism.

To verify whether Baltic-acetogens could rapidly switch to H\(_2\) after being adapted to Fe\(^0\) as the sole electron donor for eight transfers, we incubated the acetogens on H\(_2\) (after BES-inhibition of methanogens). H\(_2\)-dependent acetogenesis took 5 days to commence, similar to Fe\(^0\)-incubations (Fig. 4). Unlike a 5-day long Fe\(^0\)-dependent acetogenesis (Fig. 2f), H\(_2\)-dependent acetogenesis continued steadily for 20 days (Fig. 4). Although Baltic-acetogens were effective H\(_2\)-utilizers, the rates of abiotic H\(_2\) formation from Fe\(^0\) could not explain the tripling in electron recovery rates by acetogens on Fe\(^0\) (Fig. 2c, f). Therefore, we assumed that extracellular hydrogenases might stimulate electron uptake from Fe\(^0\) by inducing enzymatic H\(_2\)-formation and subsequently enhancing the rates of acetogenesis from Fe\(^0\). To determine if such enzymes had a stimulatory effect, we filtered the spent medium of a pre-grown Fe\(^0\)-culture into a fresh culture provided with Fe\(^0\). If the active enzymes present in the spent-filtrate stimulated the growth of acetogens then the rate of electron recovery from Fe\(^0\) into acetate would increase. Indeed, spent filtrate stimulated acetogenesis, which started 5 days earlier (Fig. 5a) than it did in Fe\(^0\)-grown or H\(_2\)-grown cultures of Baltic-acetogens. Moreover, acetate recoveries were the highest after the addition of spent filtrate (Fig. 5a) compared to the unamended community (22% increase, \(n = 10\), \(p < 0.00001\)), or unamended acetogens (7% increase; \(n = 10\), \(p < 0.02\)). These results suggest that Baltic-acetogens use an enzyme-mediated mechanism to enhance electron uptake from Fe\(^0\), similar to other acetogens [19, 55].

Like acetogens, methanogens are believed to retrieve electrons from Fe\(^0\) via an enzyme-mediated electron uptake mechanism [16, 18, 19, 64] or a poorly understood direct electron uptake system [15, 17]. Thus, to distinguish between these two mechanisms, we tested the impact of H\(_2\) and spent-media filtrate on the growth of Baltic-methanogens.

Unlike Baltic-acetogens, Baltic-methanogens could not utilize H\(_2\) for methanogenesis (Fig. 4), also their methane productivity declined after the addition of spent media filtrate (−23%; \(n = 10\); \(p < 0.03\); Fig. 5b). These results suggest that Baltic-methanogens did not use an enzyme-mediated electron uptake mechanism. These data corroborate with previously published results initiated from the same sediment and in which we observed that *Methanosarcina* was capable of mineral-mediated syntrophy independent of enzymes from spent filtrate additions [40]. Additionally, in the present study we show that Baltic-*Methanosarcina* preferred Fe\(^0\) as electron donor and were unable to consume acetate or abiotic-H\(_2\), demonstrating that they were likely retrieving electrons directly from Fe\(^0\) (Figs. 1b and 4).

**Sporomusa and Methanosarcina dominate the corrosive microbial community**

16S rRNA gene and metagenome sequence (MGS) analyses of the corrosive community identified *Proteobacteria*, *Firmicutes*, and *Euryarchaeota* (Fig. 6a, b) as representative phylotypes.
Proteobacteria clustered primarily with the genus Desulfovibrio. All Desulfovibrio sequences were most similar to *D. idahonensis* (97.5% identity; Fig. 6d) which was isolated from a metal(loid) contaminated sediment [65].

Baltic-Desulfovibrio were also related (98.5% identity) to a *Desulfovibrio* from a drinking water system contaminated by iron oxides [66]. Desulfovibrio species are capable of corrosion [5] under high-sulfate conditions.
marine environments (ca. 28 mM sulfate [67]). However, the methanogenic zone of the Baltic Sea contains no sulfate [6] and thus is a low-sulfide environment (0–2 mM [68]). Therefore, under the low-sulfate conditions in our media (ca. 0.4 mM) Desulfovibrio could only (i) use trace sulfate for its metabolism or (ii) ferment dead-biomass organics (e.g. pyruvate, fumarate) alone [69] or syntrophically [70].

Acetogens identified by MGS and 16S rRNA gene libraries belonged to the Firmicutes genera Sporomusa, Clostridium, and Acetobacterium (Fig. 6a, b, d). Spore-bearing curved rods resembling Sporomusa were visually observed in our cultures (Fig. 7a). Sporomusa dominated (>88%) the bacterial community according to 16S rRNA gene qPCR analyses, independent of the incubation period (Fig. 7j). The closest isolated relative for our Baltic-Sporomusa was S. silvacetica (97.4% identity), previously shown to be capable of electroacetogenesis on a cathode at −400 mV vs. SHE [61]. Its closest uncultured relative (99.3% identity) was a Sporomusa kenriched on a cathode (Genbank KJ600503 Fig. 6d).

Methanogens identified by MGS and 16S rRNA gene libraries belonged to Methanomicrobiia represented by the genera Methanosarcina, Methanocalculus, and Methanocella. Because the Baltic-methanogens could not utilize H2 (1.5 atm; Fig. 4) for methanogenesis, we expected Methanosarcina to be the dominant member of the methanogenic community. As such, Methanosarcina became 26 times more abundant at the end of the incubation period (day 60) than they were during the first stage of incubation (day 18; Fig. 7j). During the early stages of Fe0-dependent growth (18 days), Methanosarcina cells formed diplococci (Fig. 7d) or tetrads (Fig. 7e), but after 2 months of incubation, multicellular aggregates could be visualized by F420-autofluorescence specific for methanogens (Fig. 7g–i). No other methanogenic morphotypes were observed with F420-autofluorescence [54], indicating that Methanosarcina was the dominant methanogen.

Methanosarcina is the only known genus that includes species incapable of methanogenesis from H2 [22, 25, 26], acetate [71], or both [23, 24]. Additionally, Methanosarcina includes species capable of direct electron uptake from electrodes [29, 30] and other cells either directly [29, 31, 32], or via conductive minerals [29, 31–34, 72]. The closest relative of Baltic-Methanosarcina was the non-acetoclastic and non-hydrogenotrophic M. subterranea (Fig. 6c). The inability of their closest relative to use H2 and acetate aligns with physiological evidence that Baltic-Methanosarcina was also incapable of methanogenesis using these substrates (Figs. 1c, 4). Conclusively, Baltic-Methanosarcina was unlikely to consume acetate produced by Baltic-acetogens. Hence, our results contest previous suppositions that Methanosarcina and acetogens mainly interact syntrophically, via acetate-transfer, within a corrosive microbial community [73]. Instead, we provide evidence that Baltic-Methanosarcina were more metabolically

Fig. 7 Morphotypes and abundant phylotypes from a Baltic corrosive community. a Scanning electron microscopy (SEM) image of a spore-forming curved rod resembling a Sporomusa sporulating cell. b SEM micrograph of tetrads of cocci resembling Methanosarcina and their usual cocci aggregates. SEM was performed at the end of transfer #5 (ca. 3 months). c Epifluorescence micrographs of DAPI-stained cells detached from Fe0-granules by shaking and sonication during day 18 of transfer #10. We observed two morphotypes: a banana-shaped rod and diplococci. d The diplococci were Methanosarcina as identified by a specific probe for in situ hybridization (Cy3/red-MX821). e Baltic-Methanosarcina sometimes also formed tetrads and could never be visualized as single cells in these Fe0-dependent cultures. f Epifluorescence micrograph of DAPI-stained cells detached from Fe0-granules by vigorous shaking during day 60 of transfer #10. Only two morphotypes were observed—banana-shaped rods and cocci joined in large aggregates. g Methanosarcina formed cocci aggregates which could be detected by their natural F420-autofluorescence. No other morphotypes of methanogens could be detected. h and i Methanosarcina- aggregates with various morphologies and compactness. j Group-specific qPCR to determine the abundance of Methanosarcina and Sporomusa after 18 and 60 days of incubation on Fe0.
active on Fe$^0$ in the absence of bacterial partners (Fig. 2e). Indeed, the methanogens appeared to compete with acetogens to access Fe$^0$, since Fe$^0$-dependent methanogenesis decreased approximately threefold when acetogens were active (Fig. 2d, e).

Moreover, our results indicate that Baltic-Methanosarcina might be using a direct mechanism of electron uptake from Fe$^0$, since they could not use H$_2$, independent of its origin (Figs. 4 and 5b). Extracellular enzyme-facilitated Fe$^0$ corrosion has only been demonstrated in Methanococcus species [16, 19], while Methanosarcina species were thought to retrieve electrons from Fe$^0$ via abiotic H$_2$ uptake [21]. However, some Methanosarcina cannot use H$_2$ at all [22–26], while others have high H$_2$-uptake thresholds (296–376 nM) [27, 28]. Therefore, when using abiotic or enzymatic H$_2$ Methanosarcina should be outcompeted by strict hydrogenotrophic methanogens with low H$_2$-uptake thresholds (e.g. 6 nM for Methanobacterium formicicum) [27, 28], yet this was not the case in our enrichments indicating they may use an alternative electron uptake mechanism. Similar to other studies Methanosarcina, rather than strict hydrogenotrophic methanogens dominated the Fe$^0$ corroding community, suggesting that Methanosarcina can in fact out-compete strict hydrogenotrophic methanogens from a corrosive community. The mechanism of direct electron uptake from Fe$^0$ or any other insoluble electron donors employed by Methanosarcina is unknown. Extracelluar electron uptake in Methanosarcina has been examined recently using a comparative transcriptomics approach contrasting Methanosarcina provided either with electrons from a current-producing syntrophic partner (electrogenic Geobacter [31, 32]) or with H$_2$ from a H$_2$-producing syntrophic partner (Pelobacter) [35]. During extracellular electron uptake from an electrogenic bacterium, Methanosarcina up-regulated cell-surface proteins with redox properties, such as cupredoxins, cytochromes, and other Fe-S-proteins [35]. However, the exact role of these reductively active proteins in Methanosarcina’s extracellular electron uptake from insoluble extracellular electron donors (Fe$^0$, other cells or electrically conductive particles) remains enigmatic and requires future exploration.

Conclusion

Methanosarcina and acetogens often cohabit on the surface of corroded Fe$^0$-structures from low-sulfate environments. However, the role of Methanosarcina was assumed to be commensal, feeding on the acetate produced by acetogens. Our results demonstrate that Baltic-Methanosarcina does not establish a syntrophic partnership with acetogens based on acetate transfer as often reported. Instead, Baltic-Methanosarcina and Baltic-Sporomusa competed with one another to reclaim electrons from Fe$^0$, and each group became favored when specific inhibitors for their competitors were added to the medium. While Baltic-acetogens seem to be stimulated by enzymes/shuttles from spent filtrate, Methanosarcina were not. Moreover, Baltic-Methanosarcina were unable to utilize acetate and H$_2$ as electron donors, suggesting that they may be retrieving electrons directly via a largely unexplored mechanism.

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Author contributions PAP and A-ER designed the experiments. A-ER carried out sampling, and processing of the Baltic Sea sediment, as well as the initial inoculations and in situ hybridization experiments. BT carried out elemental identification and analyses of corrosion products. PAP carried all downstream growth experiments and analyses. PAP did all molecular experiments and analyses with support from A-ER, CRL, and OS-W. PAP and A-ER wrote the manuscript, and all authors contributed to the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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