Reduction of Fe(III) oxide by methanogens in the presence and absence of extracellular quinones

Daniel R. Bond and Derek R. Lovley
Department of Microbiology, 106 N Morrill IV N, University of Massachusetts, Amherst, MA 01003, USA.

Summary
Five methanogens (Methanosarcina barkeri MS, Methanosphaera cuniculi 1R7, Methanobacterium palustre F, Methanococcus voltaei A3 and Methanolobus vulcani PL-12/M) were investigated for their ability to reduce Fe(III) oxide and the soluble quinone anthraquinone-2,6-disulphonate (AQDS). Two species (M. barkeri and M. voltaei) reduced significant amounts of Fe(III) oxide using hydrogen as the electron donor, and 0.1 mM AQDS greatly accelerated Fe(III) reduction by these organisms. Although Fe(III) appeared to inhibit growth and methanogenesis of some strains, hydrogen partial pressures under donor-limited conditions were much lower (<0.5 Pa) in the presence of Fe(III) than in normal media (1–10 Pa) for all species except for M. vulcani. These results demonstrate that electrons were transferred to Fe(III) by hydrogen-utilizing methanogens even when growth and methanogenesis were inhibited. All species except the obligate methylotroph M. vulcani were able to reduce AQDS when their growth substrates were present as electron donors, and rates were highest when organisms used hydrogen as the electron donor. Purified soil humic acids could also be reduced by the AQDS-reducing methanogens. The ability of methanogens to interact with extracellular quinones, humic acids and Fe(III) oxides raises the possibility that this functional group of organisms contributes to Fe(III) and humic acid reduction under certain conditions in the environment and provides an alternative explanation for the inhibition of methanogenesis in some Fe(III)-containing ecosystems.

Introduction
Factors controlling the rate and extent of methane production in anaerobic sedimentary environments are of interest because methane is an important greenhouse gas, and because methane produced in deep sediments represents a significant store of utilizable energy (Kvenvolden, 1999; Hansen et al., 2000). The availability of Fe(III) as an alternative electron acceptor for microbial respiration can be a major factor controlling the amount of methane that is formed. Numerous studies have demonstrated that, when poorly crystalline Fe(III) oxides are present in sediments in which methane production is the predominant terminal electron-accepting process (TEAP), methane production is inhibited (Lovley and Phillips, 1987a; Roden and Wetzel, 1996; Frenzel et al., 1999; Yao and Conrad, 1999; Chidthaisong and Conrad, 2000). This inhibition is usually associated with a decrease in the concentrations of acetate and hydrogen, the two primary electron donors for methane production in sediments (Winfrey and Zeikus, 1979; Lovley et al., 1982; Conrad, 1999). When acetate or hydrogen is added to sediments to increase the concentrations of either of these electron donors, methane production generally resumes. These results suggest that Fe(III) is not toxic to methanogens and that the inhibition of methane production in the presence of Fe(III) results from Fe(III)-reducing microorganisms maintaining hydrogen and acetate at levels too low for methanogenesis to be thermodynamically favourable (Lovley and Phillips, 1987a).

However, in recent studies with hyperthermophilic microorganisms, it was found that washed cell suspensions of some hyperthermophilic methanogens could reduce soluble Fe(III) citrate (Vargas et al., 1998). The ability of these organisms to reduce insoluble Fe(III) oxide, the predominant form of Fe(III) in sedimentary environments (Lovley, 2000a), was not evaluated. However, most organisms that can use soluble Fe(III) as an electron acceptor can also transfer electrons onto insoluble Fe(III) oxides (Lovley, 2000b). Subsequent studies (Lovley et al., 2000) demonstrated that some of these same hyperthermophilic methanogens could transfer electrons to extracellular quinones such as humic acids and the humic acid analogue anthraquinone-2,6-disulphonate (AQDS). This has relevance for Fe(III) oxide reduction in sediments because, once extracellular quinones are reduced to the hydroquinone state, they can rapidly react abiotically with Fe(III) oxides, reducing Fe(III) to Fe(II) and regenerating the quinone form of the molecule, which can then undergo another cycle of reduction and oxidation (Lovley et al., 1996; 1998).
electron shuttling via extracellular quinones permits microorganisms to reduce Fe(III) oxide without directly transferring electrons to the Fe(III).

The purpose of this study was to determine if methanogenic microorganisms representative of those found in sediments of more moderate temperatures could reduce insoluble Fe(III) oxides, either directly or by first transferring electrons to extracellular quinones. The results demonstrate that mesophilic methanogens can reduce Fe(III) oxides and extracellular quinones. Thus, the inhibition of methane production in Fe(III) oxide-containing sediments may result, at least in part, from methanogens diverting electrons to Fe(III) and extracellular quinone reduction.

Results

Reduction of Fe(III) oxide in the presence and absence of AQDS

When *M. barkeri* was inoculated into standard growth medium with hydrogen, methane was produced over time (Fig. 1). The addition of Fe(III) oxide to this medium resulted in an increase in Fe(II) that was significantly higher than Fe(II) accumulation observed in sterile medium as the result of abiotic reactions with medium constituents (15.2 mM when corrected for sterile controls). After 7 days of incubation, non-Fe(III)-containing cultures produced 40.1 ± 2.4 mM CH₄ (mean ± SD, n = 2), whereas Fe(III)-containing cultures produced 37.5 ± 3.3 mM CH₄. Although this decrease in electron equivalents appearing as methane in Fe(III) oxide-amended cultures was small (20.8 mM), it was similar to the electron equivalents observed in Fe(II).

*M. barkeri* reduced slightly less Fe(III) (10.2 ± 0.4 mM) when methanol was provided as the electron donor. As in hydrogen-grown cultures, methane production and Fe(III) reduction proceeded simultaneously, and less methane appeared to be produced in Fe(III)-containing cultures than in control incubations as a result of diversion of electrons to Fe(III) reduction (data not shown).

Addition of 100 μM anthraquinone-2,6-disulphonate (AQDS) to hydrogen–Fe(III) oxide cultures of *M. barkeri* greatly increased the extent of Fe(III) oxide reduction (Fig. 1). The total amount of methane produced was significantly less in the AQDS–Fe(III) cultures than in cultures with Fe(III) alone. The difference in the amount of methane produced in control cultures and AQDS–Fe(III) oxide cultures was 9.8 mM, or a decrease of 78.4 mM electron equivalents, which was balanced by 70.5 mM of electron equivalents consumed in Fe(III) reduction. Surprisingly, AQDS had limited effect on methanol-grown *M. barkeri*, as only 15 mM Fe(II) was produced, compared with 10.1 mM in non-AQDS-containing cultures.

*M. barkeri* could be repeatedly transferred in both hydrogen- and methanol-containing growth medium amended with Fe(III) oxides, with similar results in each subsequent transfer. However, methane production, cell growth and Fe(III) reduction were completely inhibited when acetate was the electron donor in Fe(III)-containing growth media. Similar results (lack of methane production or growth) were observed when AQDS was added along with Fe(III), and there was only slight reduction of Fe(III) (<3 mM).

In contrast to *M. barkeri*, which reduced Fe(III) and produced methane simultaneously, the obligate hydro-
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genotroph *M. voltaei* produced methane more slowly while reducing Fe(III) oxides (Fig. 2). This pattern, of >20 mM Fe(II) accumulating before significant methanogenesis began, was observed even when cultures were repeatedly transferred in media containing Fe(III). The total amount of Fe(III) reduced by *M. voltaei* was similar to levels reduced during *M. barkeri* incubations. *M. voltaei* produced an average of 19.3 ± 0.9 mM Fe(II) after 7 days of growth with hydrogen as the electron donor. These cultures produced 3.2 mM less methane when Fe(III) was present, indicating that 25.6 mM electrons had been diverted to Fe(III) reduction. When 100 μM AQDS was included in the growth medium, there was a large increase in the extent of Fe(III) reduction (Fig. 2). Cultures containing both AQDS and Fe(III) produced 8 mM less methane than non-Fe(III)-containing cultures, corresponding to a total of 64 mM electrons diverted from methanogenesis to Fe(III) in these cultures. This value was similar to the electron equivalents consumed in Fe(III) reduction.

*M. cuniculi*, which can produce methane only by reducing methanol with hydrogen as an electron donor, produced 29.5 ± 1.1 mM Fe(II) in 7 days when hydrogen and methanol were included in the growth medium (data not shown). However, no methane production was detected in these cultures, and subsequent transfers demonstrated no activity, indicating that growth was inhibited. Similar levels of Fe(III) reduction were also observed if methanol was omitted, and only hydrogen was provided. Addition of 100 μM AQDS to *M. cuniculi* cultures containing Fe(III) did not significantly alter the rate or extent of Fe(III) reduction, and no methane production or growth was observed in the AQDS-amended cultures.

*M. palustre*, a methanogen able to utilize hydrogen and secondary alcohols as electron donors, did not demonstrate any significant Fe(III) reduction when provided with either hydrogen or 2-propanol, but this strain also could not produce methane or be repeatedly transferred in Fe(III)-containing media. In a similar manner, no significant Fe(III) reduction was observed in cultures of the obligate methanol-utilizing methanogen *M. vulcani*, and this strain grew poorly in the presence of Fe(III). The addition of AQDS did not effect the growth of these organisms.

As growth may not be necessary in order for microorganisms in sediments to have a significant impact on metal biogeochemistry (Lovley et al., 1993), the ability of cell suspensions to reduce Fe(III) oxide was also evaluated. As is typically seen with most Fe(III)-reducing microorganisms, washed cells reduced Fe(III) oxide only slowly, if at all, in short-term incubations. However, *M. barkeri* and *M. voltaei* rapidly reduced Fe(III) oxide using AQDS as an electron shuttle while simultaneously producing methane from hydrogen. As observed with growing cultures, these Fe(III)-reducing suspensions (containing Fe(III) oxides and AQDS) produced less total methane, and the decrease in methane yield could be accounted for by the number of electrons transferred to Fe(III). If the methanogenesis inhibitor 2-bromoethanesulphonic acid (BES) (50 mM) was included in these incubations to inhibit methanogenesis, Fe(III) reduction in the presence of AQDS was still observed, whereas methane production was inhibited by 96%.

Cell suspensions of *M. barkeri* grown on methanol, and provided with methanol as an electron donor, did not reduce Fe(III) using AQDS as an electron shuttle.
However, these same cells could reduce Fe(III) oxide with AQDS if hydrogen was provided. Interestingly, strains that were inhibited by Fe(III) oxide in growth media (\(M.\) cuniculi, \(M.\) palustre, \(M.\) voltaei and acetate-grown \(M.\) barkeri) produced methane in the presence of Fe(III) in washed cell suspensions. However, none of these cell suspensions reduced Fe(III) oxide under these conditions, even when AQDS was added (data not shown).

**Effect of Fe(III) oxides on hydrogen concentrations**

Thresholds for hydrogen uptake by methanogens in the presence of Fe(III) were determined as an alternative indication of electron flow to Fe(III). All of the hydrogen-utilizing methanogenic strains metabolized hydrogen down to a threshold headspace hydrogen concentration similar to previously reported studies (3–10 Pa) (Lovley, 1985; Zinder and Anguish, 1992; Zinder, 1993) (Fig. 3). \(M.\) cuniculi was able to achieve a lower threshold hydrogen partial pressure, which is consistent with the fact that reduction of methanol with one molecule of \(H_2\) (\(\Delta G^\circ = -112\ \text{kJ mol}^{-1}\) methane) is less dependent on the hydrogen concentration than the reduction of carbon dioxide with four molecules of \(H_2\) (\(\Delta G^\circ = -131\ \text{kJ mol}^{-1}\) methane).

When Fe(III) oxide was included in the medium, all strains, except for \(M.\) vulcani (which did not reduce Fe(III) in studies described above), consumed hydrogen down to 0.5–0.1 Pa \(H_2\). These levels are at least 10-fold lower than standard hydrogen thresholds reported for methanogenic bacteria (Lovley, 1985; Zinder, 1993), and the \(\Delta G^\circ\) for hydrogen-dependent methanogenesis becomes positive at these \(H_2\) concentrations (–2 to +14 kJ mol\(^{-1}\) methane, assuming 20 mM HCO\(_3\) and 0.5 atm CH\(_4\)). However, these levels of hydrogen are similar to those observed in Fe(III)-reducing cultures and environments (Lovley and Goodwin, 1988; Cord-Ruwisch et al., 1998). These results suggested that even strains that were unable to grow in the presence of Fe(III), such as \(M.\) palustre or acetate-grown \(M.\) barkeri, still passed electrons to Fe(III), when growth and methanogenesis were inhibited. The results also further confirmed that \(M.\) vulcani was unable to interact with Fe(III) oxides.

Addition of small amounts of AQDS (0.1 mM) had no significant effect on the final headspace hydrogen concentration in Fe(III) oxide media (Fig. 3), and 0.1 mM AQDS did not enhance the ability of \(M.\) vulcani to reduce the hydrogen partial pressure in Fe(III)-containing media. If excess AQDS (5 mM) was added directly to cultures, the \(H_2\) concentration declined rapidly to levels similar to those seen in Fe(III)-containing cultures (<0.5 Pa) for all strains except for \(M.\) vulcani.

Monitoring hydrogen concentrations in cultures of \(M.\) barkeri in which acetate was the electron donor suggested a mechanism for the inhibition of acetate-dependent methanogenesis in this organism (Fig. 4). When \(M.\) barkeri was inoculated into standard media containing acetate, hydrogen accumulated in the headspace (>40 Pa), and was consumed when acetate was

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**Fig. 3.** Headspace hydrogen concentrations after 7 days in substrate-limited cultures of methanogenic species growing on a variety of electron donors, and incubated without Fe(III) oxide, with 130 mM Fe(III) oxide and with Fe(III) plus 0.1 mM AQDS. Means are the result of triplicate incubations for each condition.

**Fig. 4.** Effect of Fe(III) on headspace hydrogen concentrations in acetate-grown \(M.\) barkeri cultures. Cultures were provided with acetate (5 mM), acetate plus 130 mM Fe(III) oxide or acetate, Fe(III) and 0.1 mM AQDS. Inset: expanded view of hydrogen concentrations in Fe(III)-containing cultures. Methane production was detected only in non-Fe(III)-containing cultures.
incubations (130–310 nmol CH₄/mg protein min during methanogenesis observed in similar washed cell AQDS were lower than maximal rates of electron transfer with 2-propanol. All of the rates of electron transfer to barkeri provided with acetate and 57x313) with hydrogen as the electron donor, AQDS reduction was nearly undetectable (<1 nmol AQDS/mg protein min). However, when hydrogen was added to these methanol-grown M. barkeri cell suspensions, AQDS was rapidly reduced (95 nmol AQDS/mg protein min⁻¹) (Fig. 5). The addition of hydrogen also caused AQDS reduction in acetate-grown M. barkeri. As expected, the obligate methanol-utilizing strain M. vulcani, which demonstrated no ability to reduce AQDS with methanol as an electron donor, also did not reduce AQDS when hydrogen was added. Inhibiting methane production by incubating the methanogens under CO₂-free conditions, or in the presence of BES (50 mM), had no effect on AQDS reduction when hydrogen was the electron donor. However, addition of BES inhibited acetate-dependent AQDS reduction in cell suspensions of M. barkeri by 78%.

A subset of AQDS-reducing methanogens were also tested for the ability to reduce humic acids. Electrons were transferred to soil humic acids by cell suspensions of M. barkeri, M. cuniculi (Fig. 6) and M. palustre(data not shown). Humic acid reduction rates (20–40 nmol electron equivalents/mg protein min⁻¹ for all species) were lower than rates seen with AQDS.

**Discussion**

The results demonstrate for the first time that a phylogenetic and physiological diversity of methanogens can transfer electrons to poorly crystalline Fe(III) oxide, both directly and via electron shuttling with extracellular quinones. As detailed below, the reduction of Fe(III) and extracellular quinones appears to be linked to hydrogen oxidation, rather than to methanogenesis. Although the inhibition of methane production in the presence of Fe(III) has previously been considered a result of non-methanogenic Fe(III)-reducing microorganisms outcompeting methanogens for electron donors (Lovley and Phillips, 1987a; Lovley, 2000a), the results presented here suggest that diversion of electron flow to Fe(III) reduction by methanogens, as well as direct inhibition by Fe(III)-oxide, may also be a factor.

**Implications for methanogen physiology**

The methanogens that were evaluated represent members of phylogenetically distinct orders that utilize very different electron transport systems for energy generation (Deppenmeier et al., 1996; Deppenmeier et al., 1999). For instance, members of the Methanosarcinales, of which M. barkeri, M. vulcani and M. cuniculi are members, possess cytochromes, while the obligate hydrogenotrophic Methanomicrobiales (e.g. M. palustre) and Methanococcales (e.g. M. voltaei) do not. In addition, the fundamental hydrogenases involved in the initial and

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**Table 1. Rates of AQDS reduction by washed cell suspensions of methanogens provided with a variety of electron donors.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Donor</th>
<th>AQDS reduction rate (±SD) (nmol AQDS/mg protein min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanosarcina barkeri</td>
<td>H₂</td>
<td>180 (±35)</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>23 (±7)</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Methanosphaera cuniculi</td>
<td>H₂</td>
<td>37 (±6)</td>
</tr>
<tr>
<td>Methanobacterium palustre</td>
<td>H₂</td>
<td>150 (±19)</td>
</tr>
<tr>
<td></td>
<td>2-Propanol</td>
<td>9.3 (±2.5)</td>
</tr>
<tr>
<td>Methanococcus voltaei</td>
<td>H₂</td>
<td>245 (±13)</td>
</tr>
<tr>
<td>Methanolobus vulcani</td>
<td>Methanol</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
final steps of methanogenesis differ greatly across these orders. Quinones have not been found in methanogens, but phenazine compounds have recently been shown to fulfill a quinone-like role in electron transfer in Methanosarcinales (Abken et al., 1998). Despite these differences, all of the methanogens which had a capacity to oxidize hydrogen also had the capacity to reduce Fe(III) oxide and extracellular quinones.

Common to all methanogen strains investigated was the observation that hydrogen oxidation, rather than methanogenesis per se, was linked to reduction of Fe(III) and extracellular quinones. These results suggest that a hydrogenase may be reducing these compounds, similar to the common observation that methanogenic hydrogenase...
nases can reduce compounds such as viologen dyes. All methanogens metabolized hydrogen to significantly lower levels when Fe(III) or AQDS was provided as an alternative electron acceptor, even when the presence of Fe(III) inhibited methane production (e.g. *M. palustre*). Furthermore, AQDS was still reduced when methanogenesis was inhibited with BES or by CO₂ deprivation. Methanogens that were grown and incubated under conditions in which hydrogen was not added (e.g. *M. barkeri* with methanol, or *M. vulcani* with methanol) also showed little or no activity with either Fe(III) or AQDS, even though active methanogenesis was observed.

Further evidence for a role of hydrogen in Fe(III) and AQDS reduction was obtained when H₂ was added to methanol- and acetate-grown *M. barkeri* cells. In these incubations, the rate of AQDS reduction was very slow until H₂ was added, at which point AQDS reduction dramatically increased, suggesting that these washed cell suspensions had not established hydrogen levels necessary for an existing hydrogenase to utilize for quinone or Fe(III) reduction. A similar H₂ addition to the related *M. vulcani*, which could not reduce AQDS using methanol as a donor, had no effect. As this organism is an obligate methanol oxidizer that does not utilize hydrogen in its metabolism, this again implicates hydrogenases as a component in quinone and Fe(III) reduction by methanogens.

While methanogens were long believed to be strict anaerobes which require a low redox potential, cell suspensions containing oxidized AQDS, oxidized humic acids or Fe(III) oxides rapidly initiated methanogenesis in all strains. This is in agreement with others who have found that methanogenesis can occur in oxidized, anoxic soils (Roy et al., 1997; Yao and Conrad, 1999). Similar to other Fe(III)-reducing microorganisms, in washed cell suspensions, only quinone electron acceptors were utilized, and no direct Fe(III) reduction was detected. Only when AQDS was present as an electron shuttle could two species (*M. barkeri* and *M. voltae*) reduce Fe(III) in washed cell suspensions.

**Environmental implications**

There are likely to be many instances in which Fe(III) oxides become available in sediments which also contain significant numbers of methanogens. For example, in rice paddies and wetlands, when flooded methanogenic soils are subsequently dried and exposed to air, Fe(II) in the soil is oxidized back to Fe(III). Methanogens survive in such soils (Fetzer and Conrad, 1993; Frenzel et al., 1999; Yao and Conrad, 1999) and become metabolically active when the soils are reflooded and anaerobic conditions develop. Oxygen introduced into anoxic sediments along the rhizosphere of aquatic plants may generate Fe(III)-oxide rich zones (Frenzel et al., 1999; King and Garey, 1999). Bioturbation is also likely to mix Fe(III) oxide-containing surficial sediments with methanogenic sediments in some environments.

Previous studies which demonstrated the inhibition of methane production when poorly crystalline Fe(III) oxides were introduced into methanogenic sediments suggested that this inhibition resulted from Fe(III)-reducing microorganisms not capable of methane production, outcompeting methanogens for important electron donors, such as hydrogen and acetate (Lovley and Phillips, 1987a). This hypothesis was subsequently supported by pure culture studies which demonstrated that hydrogen-oxidizing Fe(III) reducers had lower thresholds for hydrogen than methanogens. However, these previous studies did not consider the possibility that methanogens might be able to reduce Fe(III) as well. The results presented here demonstrate that, when Fe(III) is available, many methanogens divert electrons to Fe(III), and can continue to consume hydrogen for the reduction of Fe(III) at hydrogen levels that are too low to support methane production. This suggests that at least a portion of the inhibition of methane production when Fe(III) is introduced into methanogenic sediments results from methanogens diverting electron flow to Fe(III) reduction. A similar diversion of electron flow to Fe(III) reduction without any apparent energy yield is also observed in sulphate-reducing microorganisms (Coleman et al., 1993; Lovley et al., 1993). In addition, the presence of Fe(III) in growth media appeared to be toxic to some strains, an effect which could further account for the inhibition observed in soils.

These results also suggest why acetate-dependent methanogenesis in Fe(III)-containing soils is suppressed, even when acetate levels are high enough to support such activity (Achtnich et al., 1995; Conrad, 1999). During acetoclastic methanogenesis, *M. barkeri* produces hydrogen via oxidation of the carbonyl group of acetyl CoA (Lovley and Ferry, 1985; Zinder and Anquish, 1992; Meuer et al., 1999). This evolved hydrogen represents the only reducing equivalents available for reduction of the CH₃ group of acetate, and must be recaptured. If these electrons are instead diverted to Fe(III) reduction, then methanogenesis from acetate by this group of organisms would be impossible.

The finding that methanogens can reduce AQDS contrasts with conclusions from a recent report (Cervantes et al., 2000) which studied the reduction of AQDS by suspensions of methanogenic sediments and fermenter samples amended with high concentrations (20 mM) of AQDS. Because BES was found to have little effect on AQDS reduction, and AQDS was reduced in the absence of methanogenesis, it was proposed that methanogens were not involved in quinone reduction (Cervantes et al., 2000).
Furthermore, it was found that methanogenic consortia fed methanol were unable to reduce AQDS, and that acetate-oxidizing consortia reduced AQDS poorly. These results, such as the ability of BES-inhibited methanogens to reduce AQDS, and the fact that methanol cannot serve as an electron donor, are all in agreement with the studies reported here, using pure cultures of methanogens, and suggest that methanogens should be considered potential members of the quinone-reducing population.

In summary, these studies suggest that many, but not all, methanogens can reduce poorly crystalline Fe(III) oxide directly, and can also reduce extracellular quinones, such as AQDS and humic acids, which can serve as electron shuttles to promote Fe(III) oxide reduction. Although the reduction of Fe(III) and extracellular quinones appears to be related to hydrogen oxidation, the enzymatic mechanisms for the reduction of the extracellular electron acceptors are not yet known and warrant further investigation.

Experimental procedures

Organisms and growth conditions

*Methanosarcina barkeri* MS (Oregon Collection of Methanogens (OCM) 38), *Methanosphaera cuniculi* 1R7 (OCM 183), *Methanobacterium palustre* F (OCM 238), *Methanococcus voltaei* A3 (OCM 197) and *Methanolobus vulcani* PL-12/M (OCM 157) were obtained from the Oregon Collection of Methanogens. Media for *M. barkeri, M. cuniculi* and *M. palustre* contained (per litre) 0.2 g MgSO4·7H2O, 0.05 g CaCl2·2H2O, 0.5 g KH2PO4, 0.5 g NaCl, 1 g NH4Cl, 0.41 g sodium acetate, 0.5 g yeast extract, 0.5 g Peptone (Difco), 0.4 g cysteine, 2 g NaHCO3, 10 ml of OCM trace mineral mix (Boone et al., 1989) and 10 ml of a standard vitamin mix (Lovley and Phillips, 1988). *M. voltaei* and *M. vulcani* were grown in the same media, with salts added to give a final concentration of (per litre) 29.2 g NaCl, 1.67 g MgCl2·6H2O and 0.5 g KCl (MgCl2 was added after autoclaving). Media were sparged with O2-free N2/CO2 (80:20) and adjusted to pH 6.8 before being anaerobically dispensed. *M. cuniculi* and *M. vulcani* were supplemented with 0.25 mg ml⁻¹ coenzyme M, *M. palustre* was supplemented with 50 μM sodium tungstate, and all media received 0.5 mM Na2S immediately before use. Where indicated, H2 was produced by replacing the headspace with H2/CO2 and methanol and 2-propanol were added to a final concentration of 0.5%, or acetate was added at 50 mM. All cultures were grown at 37°C with gentle shaking. All cultures were maintained on their electron donors for at least five transfers before using cells in Fe(III) or AQDS reduction experiments.

AQDS reduction

Actively growing cultures were centrifuged twice at room temperature (20°C) for 10 min at 10 000 g. Supernatants were removed under a stream of O2-free N2/CO2 (80:20), and pellets resuspended in a pH 6.8 anoxic buffer containing (per litre) 0.2 g MgSO4·7H2O, 0.05 g CaCl2·2H2O, 0.5 g KH2PO4, 0.5 g NaCl and 2 g NaHCO3 (with appropriate salt additions in the case of *M. voltaei* and *M. vulcani*). To limit abiotic reduction of quinones and metals, sulphide was not added, and cysteine was added at a concentration of 0.025 g l⁻¹. Cells were resuspended to a final optical density (450 nm) of 0.1–0.4, allowed to starve at 37°C on a reciprocating shaker for 30 min, and dispensed into anaerobic pressure tubes. This starvation step was found to diminish reduction of AQDS in control incubations containing no electron donor. Anthraquinone-2,6-disulphonate (ADQS) was added to a final concentration of 2 mM, along with electron donors. AQDS reduction was followed by monitoring absorbance at 450 nm in a Spectronic Genysys 5 spectrophotometer. Values were corrected for light scattering by cells using tubes containing cells and electron donor but lacking AQDS. Concentrations of reduced AQDS were calculated from standards produced by reducing AQDS with H2 over a Pd catalyst (Lovley et al., 1999).

Humics reduction

Cells were washed and starved as described above (except that the final protein concentration was fivefold higher), injected into anaerobic pressure tubes containing purified soil humics (International Humic Substances Society, University of Minnesota, St. Paul, MN, USA) to a final concentration of 2 g l⁻¹ humics, supplied with appropriate electron donors, and returned to a 37°C reciprocating shaker. Samples were anaerobically filtered through 0.2 μm filters into anaerobic tubes containing enough Fe(III) citrate to give a final concentration of 2 mM Fe(III), and incubated at room temperature for 15 min. Samples of this mixture were analysed for Fe(II) by adding to a ferrozine–Hepes reagent (Lovley and Phillips, 1987b) and measuring absorbance at 562 nm. Samples added to Hepes alone were used to correct for absorbance by humic acids (Lovley et al., 1999).

Fe(III) oxides

Fe(III) oxide was prepared as previously described (Lovley and Phillips, 1986). When included in cell suspensions, it was added from a concentrated stock to a final concentration of 130 mM. When included in growth media, it was added prior to autoclaving to a concentration of 130 mM Fe(III), and cysteine was added after autoclaving along with sulphide. These additions, plus non-specific reducing agents in the media, produced 7–8 mM Fe(II) in the absence of cells. Blank tubes containing Fe(III) were included in incubations, and this abiotic level of Fe(III) reduction was subtracted from all reported values. Fe(II) was assayed by digesting subsamples in 0.5 M HCl for 24 h, adding this digest to a ferrozine reagent and filtering through a 0.2-μm filter prior to determination of absorbance.

Hydrogen threshold measurements

Serum bottles containing 25 ml of standard growth medium were amended with 5 mM AQDS, 130 mM Fe(III) oxide or
0.1 mM AQDS + 130 mM Fe(III) as indicated. Electron donors were provided at levels equal to 5% of what was normally used for growth, and a 20% inoculum from an actively growing culture was added to each bottle. Bottles were shaken at 37°C for 7 days, at which point headspace samples (1.5 ml) were withdrawn for hydrogen analysis. Hydrogen was injected onto a 0.5 ml sampling loop, separated on a Supelco 100/120 Carbosieve S-II column at room temperature with N₂ gas as the carrier, and detected using a reduction gas analyser (RGD2, Trace Analytical, Menlo Park, CA, USA).

**Other measurements**

Methane in headspace was analysed with a Hewlett-Packard 5890 Series II gas chromatograph equipped with a HP-624 capillary column, and operated at a temperature of 100°C. Methane concentrations are expressed as mmol per litre of growth medium (mM). Protein concentrations of all washed cell suspensions were measured using the bicinchoninic acid method using reagents obtained from Sigma Chemical Company (St. Louis, MO, USA).

**References**


