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Bacterial Methanogenesis and Growth from CO_2 with Elemental Iron as the Sole Source of Electrons

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Previous studies of anaerobic biocorrosion have suggested that microbial sulfur and phosphorus products as well as cathodic hydrogen consumption may accelerate anaerobic metal oxidation. Methanogenic bacteria, which normally use molecular hydrogen (H_2) and carbon dioxide (CO_2) to produce methane (CH_4) and which are major inhabitants of most anaerobic ecosystems, use either pure elemental iron (Fe^0) or iron in mild steel as a source of electrons in the reduction of CO_2 to CH_4. These bacteria use Fe^0 oxidation for energy generation and growth. The mechanism of Fe^0 oxidation is cathodic depolarization, in which electrons from Fe^0 and H^+ from water produce H_2, which is then released for use by the methanogens; thermodynamic calculations show that significant Fe^0 oxidation will not occur in the absence of H_2 consumption by the methanogens. The data suggest that methanogens can be significant contributors to the corrosion of iron-containing materials in anaerobic environments.

CORROSION OF METALS IS A SERIOUS economic problem. A significant amount of corrosion, particularly under anaerobic conditions, is thought to be mediated by microorganisms (*1–8*). The sulfate-reducing bacteria are widely regarded as the chief agents of biocorrosion in anaerobic environments. The corrosive activities of these organisms are due in part to their ability to form corrosive hydrogen sulfide (*1–3, 5–8*) and reduced phosphorus compounds (*4, 9*). In addition, these bacteria are thought to cause corrosion by cathodic depolarization (Fig. 1), a mechanism first proposed by von Wolzogen Kühr and van der Vlugt (*10*). According to this theory, the bacteria accelerate the anodic dissolution of metal by using hydrogen (formed from water-derived protons and cathode-derived electrons) through their hydrogenase enzymes. The reducing equivalents so generated are used in the dissimilatory reduction of sulfate to hydrogen sulfide:

$$8[H] + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_2O \qquad \Delta G^{0'} = -152\ \text{kJ} \qquad (1)$$

[H] is used to describe hydrogen species of unknown structure, either as bound atomic H or H_2; $\Delta G^{0'}$ is the standard free energy change at *p*H 7. Recent work with hydrogenase-positive *Desulfovibrio* strains showed that iron was a partial source of the electrons involved in sulfate reduction; however, iron did not serve as a sole electron donor, since no sulfide was formed in the absence of lactate (*11*). Other workers have suggested that [H] is derived from a cathode of ferrous sulfide precipitated onto the metal surface (*7, 12*); this temporary cathode is thought to be continually regenerated by bacterial hydrogen removal (*7, 8*). Despite wide acceptance of the cathodic depolarization concept, there have been few unequivocal experimental demonstrations of its occurrence (*13*). There is no evidence that this phenomenon is coupled to microbial growth [except where electrically generated cathodic hydrogen supported the growth of sulfate-reducing bacteria (*14*)].

The requirement for a hydrogenase in the cathodic depolarization mechanism suggested to us that other hydrogen-using bacteria might perform cathodic depolarization in the presence of the proper electron acceptor and might couple the energy generated to bacterial growth. Non-sulfate-reducing bacteria that oxidize hydrogen would also be a cleaner experimental system for studying cathodic depolarization because problems with measuring growth, metal dissolution, and Fe^{2+} production in the presence of large amounts of precipitated sulfides could be avoided. In this report we show that methanogenic bacteria, which grow through anaerobic respiration, normally as in Eq. 2, are capable of growth and methane production with metallic iron as sole electron source:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^{0'} = -139\ \text{kJ} \qquad (2)$$

To our knowledge, this is the first demonstration of Fe^0 as an energy source for growth of any organism and thus represents a novel type of chemolithotrophic energy metabolism.

When mid-logarithmic stage cells of *Methanosarcina barkeri* were examined for their ability to oxidize Fe^0, H_2 was replaced

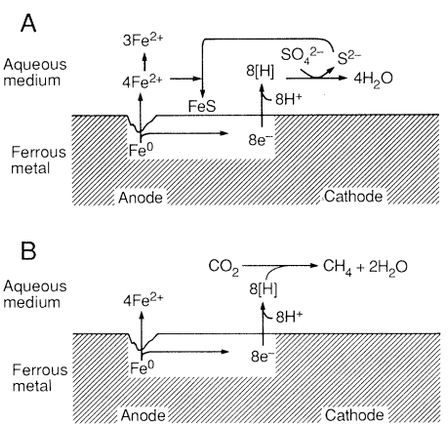

Fig. 1. (**A**) Schematic illustration of cathodic depolarization reactions proposed for corrosion of ferrous metals by sulfate-reducing bacteria according to the classical mechanism of von Wolzogen Kühr and van der Vlugt (*10*). The form of "hydrogen" is not specified. (**B**) A similar mechanism for cathodic depolarization by methane-producing bacteria.

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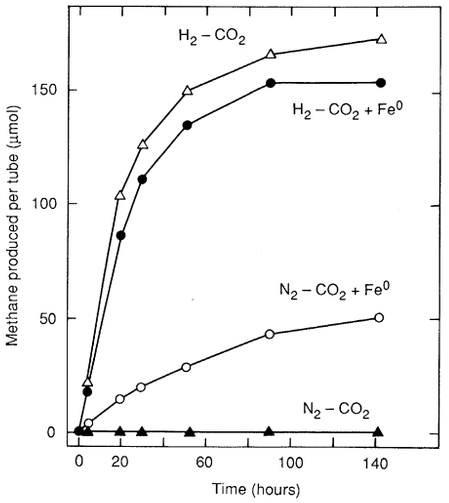

Fig. 2. Methanogenesis by suspensions of *Methanosarcina barkeri* with either H_2 or Fe^0 as electron donor. Methane formation from CO_2 as a function of time is shown for the following cases: no Fe^0 or H_2 (▲); Fe^0 but no H_2 (○); H_2 but no Fe^0 (△); Fe^0 and H_2 (●). Components of the medium and all procedures were as described in the legend to Table 1. All data points shown are the mean of triplicate tubes and are representative of those obtained in two independent experiments; the correlation coefficient was 0.82 ± 0.10.

with Fe^0 as an electron donor in methanogenesis (Fig. 2). Methanogenesis by these cells from H_2 and CO_2 was not significantly inhibited by powdered iron. When both Fe^0 and H_2 were absent, virtually no CH_4 was produced. Five different methanogens produced methane from Fe^0 and CO_2, as shown in Table 1. No CH_4 was produced in control tubes with heat-killed cells or uninoculated medium with Fe^0 and CO_2 present. Moreover, for tubes inoculated (10% inoculum) with *Methanococcus thermolithotrophicus*, 1/33 the amount of CH_4 was produced from $Fe^0 + CO_2$ when 15 μ*M* 2-bromoethanesulfonate (a highly effective inhibitor of methanogens) was added as compared to tubes with no inhibitor.

The reaction that these organisms use most likely has the stoichiometry of Eq. 3

$$8H^+ + 4Fe^0 + CO_2 \rightarrow CH_4 + 4Fe^{2+} + 2H_2O \quad \Delta G^{0'} = -136 \text{ kJ} \quad (3)$$

and has a free energy change very similar to that of methanogenic H_2 oxidation (Eq. 2).

However, anodic dissolution, when coupled only to H_2 production as shown in Eq. 4

$$4Fe^0 + 8H^+ \rightarrow 4Fe^{2+} + 4H_2 \quad \Delta G^{0'} = +3.5 \text{ kJ} \quad (4)$$

is not energetically favored. The further oxidation of Fe^{2+} as in Eq. 5,

$$8Fe^{2+} + CO_2 + 8H^+ \rightarrow 8Fe^{3+} + CH_4 + 2H_2O \quad \Delta G^{0'} = +774 \text{ kJ} \quad (5)$$

is also unfavorable. Indeed, when *M. thermolithotrophicus* cells were incubated with ferrous sulfate in the place of Fe^0, no CH_4 production was detected.

The favorability of Eq. 3 suggested that iron oxidation might be coupled to cell growth. We examined this possibility by using *M. thermolithotrophicus*, as shown in Table 2. Growth occurred under an atmosphere of N_2 and CO_2 and in the presence of Fe^0, as measured by both protein production and cell number, whereas no increase in these parameters occurred in bottles that lacked Fe^0. Growth under H_2 and CO_2 (but not methanogenesis) was inhibited significantly in the presence of Fe^0, possibly as a result of the production of Fe^{2+}. In bottle experiments with mild steel (1020 carbon steel, approximately 0.2% carbon) as an Fe^0 source and in the absence of H_2, both cell protein and CH_4 increased with time but did not increase at all when the steel was absent (Fig. 3). Thus, *M. thermolithotrophicus* can grow, albeit slowly (doubling time of 5 to 10 hours), by using Fe^0 (either as powdered iron or as steel pellets) as the sole electron donor. The reduced rate of methanogenesis from steel may be due to the low surface area of the pellets. Further experiments showed that *Methanobacterium thermoautotrophicum* can also grow with Fe^0 in mild steel as the sole electron donor.

Equation 3 predicts that Fe^{2+} is a product of methanogenesis. This prediction was ver-

Table 1. Use of elemental iron as an electron donor for methanogenesis from CO_2 by several methanogenic bacteria. Values shown are the mean ± SD of triplicate tubes and are representative of those obtained in two to three independent experiments; replicate runs that gave the same value are shown without the SD. Cells were grown on H_2 and CO_2 in 500-ml stoppered serum bottles as described in (*17–27*). The experiments were performed in serum tubes (*18, 19*) which were gassed with N_2 and CO_2 (4:1 volume ratio) and autoclaved; 0.05 g of elemental iron powder (Mallinckrodt Chemical Works, St. Louis, Missouri) was added (when needed) prior to gassing and sterilization. Mid-logarithmic stage cells were then transferred from the bottles into the tubes in 5-ml quantities. The tubes were flushed out thoroughly and pressurized to 1.4 atm with N_2 and CO_2 or H_2 and CO_2 (4:1 volume ratios); sterile anaerobic sulfide was readded to a final concentration of 1.0 m*M* after flushing. The *p*H in the tubes after such processing was the same as that specified in (*17–27*) for routine growth of cultures. Methane was measured by gas chromatography (*19*).

Organism	Incubation period (hours)	Final CH_4 production (micromoles per tube) with the following electron donors			
		None	Fe^0	H_2	$Fe^0 + H_2$
Methanococcus thermolithotrophicus	14	0.5 ± 0.1	20.6 ± 0.8	149.6 ± 8.6*	162.0 ± 5.5*
Methanobacterium thermoautotrophicum	47	0.1	37.8 ± 2.6	176.0 ± 5.0	169.0 ± 1.1
Methanosarcina barkeri	141	1.0 ± 0.3	50.8 ± 2.9	172.0 ± 3.5	155.7 ± 3.3
Methanobacterium bryantii	52	0.5	24.2 ± 3.7	186.5 ±	175.7 ± 12.0
Methanospirillum hungatei	141	1.0 ± 0.2	35.2 ± 3.9		

*These values were obtained after 8 hours of incubation.

Table 2. Methanogenesis and growth (as measured by protein concentration and direct cell count) by *Methanococcus thermolithotrophicus* with Fe^0 powder or H_2 as the source of electrons. Values shown are the mean ± SD of duplicate bottles and are representative of those obtained in two independent experiments; replicate runs that gave the same value are shown without the SD. Medium and anaerobic procedures were as described in (*17–27*). Medium was dispensed in 50-ml amounts into 250-ml serum bottles; Fe^0 power was added (0.5 g per bottle), and the bottles were gassed with N_2 and CO_2 (4:1 volume ratio) and autoclaved. Sterile anaerobic sulfide was added (final concentration 1 m*M*) after the bottles were cooled; 8 ml of inoculum was then added. Methane was determined as described in the legend to Table 1; 1-ml culture samples were removed for Bradford protein assay (*29*) and direct cell count measurements (Petroff-Hausser counter) during incubation.

Time (hours)	CH_4 (micromoles per bottle)	Protein (μg/ml)	Cell count ($\times 10^6$/ml)
	N_2 and CO_2		
0	3.2 ± 0.1	4.0	15 ± 2
2.5		4.5 ± 0.7	15 ± 1
4.5	12.4 ± 0.9	4.4 ± 0.9	12 ± 1
8	12.9 ± 0.9	4.0	8 ± 2
	N_2 and CO_2 + Fe^0		
0	3.8 ± 0.1	4.5 ± 0.7	14 ± 4
2.5	405 ± 35	6.5 ± 0.7	25 ± 1
4.5	492 ± 72	8.0	38 ± 2
8	584 ± 33	9.5 ± 0.7	34 ± 4
	H_2 and CO_2		
0	3.3 ± 0.2	4.5 ± 0.7	15 ± 1
2.5	1251 ± 48	51.5 ± 5.0	113 ± 4
4.5	1559 ± 18	110	323 ± 16
8	1701 ± 15	137 ± 5	
	H_2 and CO_2 + Fe^0		
0	3.9 ± 0.2	4.0	14 ± 3
2.5	1341 ± 10	31.5 ± 2.1	78 ± 6
4.5	1548 ± 9	64.2 ± 3.5	15 ± 4
8	1681 ± 13		

ified by the assay of Fe^{2+} during growth, as shown in Fig. 3. Bottles that contained the steel pellet produced Fe^{2+} concurrently with CH_4; after 73 hours of incubation, a Fe^{2+} to CH_4 ratio of 3.5 was obtained, which is near the stoichiometry of 4.0 predicted by Eq. 3. No Fe^{2+} production was detected in uninoculated bottles (all other conditions being the same as in the inoculated bottles) containing a steel pellet.

The cathodic depolarization theory of iron corrosion originally proposed did not distinguish between atomic ([H]) or molecular (H_2) hydrogen as reaction products (*10*). The "hydrogen" once formed could be used by sulfate-reducing bacteria to produce sulfide, as shown in Eq. 1 and Fig. 1. These workers also postulated that methanogens (as described in Eq. 3) could use [H] from the metal surfaces (*5, 15*). Since that time a variety of papers have appeared on this subject, but the theory remains controversial and, to our knowledge, no work has been reported on methanogens (*1–15*). Although earlier investigators reported that hydrogenase was needed for corrosion to occur through this mechanism, more recent work (*16*) showed that hydrogenase levels have no effect on the corrosion rate.

For our methanogen work, two possible mechanisms could be responsible: (i) the Fe^0 could be metabolized by a methanogen enzyme, or (ii) cathodic depolarization could occur with the methanogens consuming either [H] on the metal surface or H_2 released from the metal. We conducted the following two-bottle experiment [anaerobic conditions, *p*H and CO_2 content as described in (*17–27*)]: bottle 1 contained medium under N_2 and CO_2 and 0.5 g of iron powder, and bottle 2 contained medium under N_2 and CO_2 and an inoculum of *M. barkeri*; the two bottles were connected by a short section of stainless steel tubing (through the rubber stoppers on top) and were gently shaken upright at 37°C. After 142 hours of incubation, growth [absorbance at 600 nm (A_{600}) = 0.33] and methanogenesis [177 μmol of CH_4 (gas phase)] occurred in bottle 2, whereas no growth (A_{600} = 0.03) or methanogenesis [1.0 μmol of CH_4 (gas phase)] was detected in the control cultures with no Fe^0 added to bottle 1 (other conditions being the same as in the test cultures). Thus we conclude that chemical oxidation of Fe^0 by cathodic depolarization occurs and that the methanogens use the H_2 produced. This mechanism, which allows the otherwise unfavorable Fe^0 oxidation to occur by continual removal of H_2 so that its pressure is kept low, is similar to the way in which methanogens allow syntrophic bacteria to metabolize short-chain fatty acids (*28*). This experiment strongly suggests the occurrence of microbially assisted cathodic depolarization and is the first evidence that the hydrogen species produced can be H_2 that is released from the metal surface.

Calculations from the data in Fig. 3 show a mild steel corrosion rate of 79 mg per square decimeter per day (mdd). This rate is high compared to values of 1.3 to 21.0 mdd reported in studies with pure cultures of *Desulfovibrio* (*6, 11, 13*) but similar to one other report of 95 mdd (*8*). Our work suggests that methanogens could contribute significantly to metal corrosion in anaerobic areas (for example, metal objects buried in soil or sediment, submerged in water, or inside containers such as anaerobic digestors). In these systems H_2 is often present in only low levels and CO_2 is abundant, so that Fe^0 could serve as a significant electron donor.

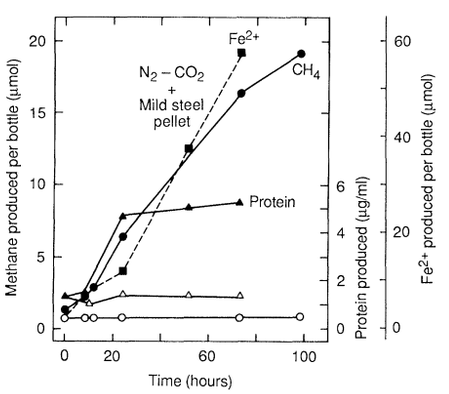

Fig. 3. Methanogenesis, protein production, and Fe^0 oxidation for production of Fe^{2+} by cultures of *Methanococcus thermolithotrophicus* with Fe^0 (steel pellet) used as the electron donor: methane production from CO_2 in cultures with CO_2 and mild steel (●) and CO_2 alone (○); formation of Fe^{2+} in cultures with CO_2 and mild steel (■); production of protein in cultures with CO_2 and mild steel (▲) and with CO_2 alone (△). There was no Fe^{2+} formation in the cultures that lacked mild steel. The experiment was conducted in 250-ml bottles as described in the legend to Table 2. Mild steel (0.53 g per bottle, surface area 1.57 cm^2) was used as the Fe^0 source. Fe^{2+} was measured as described previously (*30*). All data points shown are the mean of duplicate bottles and are representative of those obtained in two independent experiments; the correlation coefficient for CH_4, Fe^{2+}, and protein data was 0.95 ± 0.07 for the mild steel–containing experiments and 0.32 ± 0 for the experiments with no steel.

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17. Cells were grown on H_2 and CO_2 by using the anaerobic techniques described by Balch and Wolfe (*18*) and Daniels *et al.* (*19*). *Methanococcus thermolithotrophicus* [(*20*); provided by K. O. Stetter] was grown at 64°C in the medium described previously (*19*) except that NH_4Cl and $CaCl_2 \cdot 2H_2O$ were added at concentrations of 8.41 m*M* and 0.24 m*M*, respectively, and a vitamin mix (*21*) was added at 10 ml/liter. *Methanobacterium thermoautotrophicum* strain Marburg [(*22*); provided by G. Fuchs] was grown at 64°C in the medium described previously (*19*) except that the mixture of trace elements as described for *M. thermolithotrophicus* and vitamin mix was added as given above. *Methanobacterium bryantii* strain M.O.H. [(*23*); provided by G. D. Sprott] was grown at 37°C in a medium similar to that described (*24*); it consisted of the following components in distilled and deionized water: K_2HPO_4 (3.44 m*M*), KH_2PO_4 (4.41 m*M*), $MgCl_2 \cdot 6H_2O$ (0.31 m*M*), $CaCl_2 \cdot 2H_2O$ (0.22 m*M*), NaCl (5.13 m*M*), sodium acetate (5.12 m*M*), NH_4Cl (24.3 m*M*), and resazurin (0.003 m*M*); vitamin mix was added as given above; and trace elements were added as described for *M. thermolithotrophicus* (*19*). *Methanosarcina barkeri* strain 227 [(*25*); provided by S. H. Zinder] was grown at 37°C in the medium described previously (*26*) except that the composition of trace elements and vitamin mix was as given above for *M. thermolithotrophicus*. *Methanospirillum hungatei* strain GP1 [(*27*); provided by G. D. Sprott] was grown at 37°C as described in (*19*). Medium *p*H was 6.5 for *M. thermolithotrophicus* and *M. bryantii*, 6.8 for *M. barkeri*, and 7.1 for *M. thermoautotrophicum* and *M. hungatei*. The *p*H was adjusted according to the technique described previously (*19*) by the addition of Na_2CO_3 while thoroughly bubbling the medium with N_2 and CO_2 (4:1 volume ratio); the *p*H remained within ±0.1 of the initially adjusted value after gassing and regassing of medium. Na_2S was added at a concentration of 1.0 m*M* in all cases.
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