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Kinetic Analysis of Competition Between Sulfate Reducers and Methanogens for Hydrogen in Sediments†

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The competition between sulfate-reducing and methanogenic bacteria for hydrogen was investigated in eutrophic lake sediments that contained low in situ sulfate concentrations and in sulfate-amended sediments. Sulfate reduction and methane production coexisted in situ in lake surface sediments (0 to 2 cm), but methane production was the dominant terminal process. Addition of 10 to 20 mM sulfate to sediments resulted in a decrease in the hydrogen partial pressure and a concomitant inhibition of methane production over time. Molybdate inhibition of sulfate reduction in sulfate-amended sediments was followed by an increase in the hydrogen partial pressure and the methane production rate to values comparable to those in sediments not amended with sulfate. The sulfate reducer population had a half-saturation constant for hydrogen uptake of 141 pascals versus 597 pascals for the methanogen population. Thus, when sulfate was not limiting, the lower half-saturation constant of sulfate reducers enabled them to inhibit methane production by lowering the hydrogen partial pressure below levels that methanogens could effectively utilize. However, methanogens coexisted with sulfate reducers in the presence of sulfate, and the outcome of competition at any time was a function of the rate of hydrogen production, the relative population sizes, and sulfate availability.

It is generally considered that sulfate-reducing bacteria (SRB) can inhibit the activity of methanogenic bacteria (MB) when millimolar quantities of sulfate are present. Thermodynamic calculations can be used to predict the exclusion of methane production in sulfate-containing sediments (8, 14, 29). However, it is invalid to argue that a reaction that is more thermodynamically favorable will exclude another reaction that is also thermodynamically favorable (15). Therefore, MB must be inhibited by toxic metabolites, the lack of methane precursors, or required growth factors in the presence of sulfate. The prevalent conclusion is that SRB inhibit MB by outcompeting them for hydrogen and acetate (1, 2, 6, 14, 18, 21, 29), but the mechanism(s) for this have not been elucidated. MB are frequently present in sulfate-containing sediments and have the potential to consume methane precursors as evidenced by methane production when sulfate reduction is inhibited or when hydrogen or acetate is added to the sediments (2, 21, 26, 29). Our working hypothesis was that SRB have a higher affinity for hydrogen and acetate than MB, which enables SRB to maintain the pool of these substrates at concentrations too low for MB to effectively utilize when sulfate is not limiting to SRB. The studies reported here concentrated on the competition for hydrogen since acetate-utilizing MB are generally absent in natural sediments in which SRB effectively outcompete MB (8, 19, 26), and the ultimate competition is thus for hydrogen.

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MATERIALS AND METHODS

Measurements of in situ rates. Sediments were collected during summer stratification from two sites in Wintergreen Lake, a eutrophic lake located in southwestern Michigan. During summer stratification the sediments at the profundal site, site A, lie below an anaerobic, sulfate-depleted hypolimnion (sulfate concentration range, 30 to 160 μM), whereas those at the depth of the thermocline, site B, have oxygen (range, 1 to 4 mg of oxygen per liter) and sulfate (180 μM) in the overlying water (16, 17).

Sulfate reduction rates were measured by the direct injection method of Jørgenson (10) as described in detail by King and Klug (11). Briefly, 10 μl of carrier-free $^{35}SO_4^{2-}$ (1 μCi) was injected into sediment cores incubated at in situ temperatures. The incubation was stopped by quick freezing. The $^{35}S^{2-}$ produced was distilled, trapped, and quantified by liquid scintillation counting. Sulfate reduction rates were calculated by multiplying the rate of conversion of $^{35}SO_4^{2-}$ to $^{35}S^{2-}$ by the in situ sulfate pool. Interstitial water was collected with dialysis samplers (17) and analyzed for sulfate turbidimetrically (28).

Methane production was measured on 5-ml subcores taken through ports in cores (7-cm inner diameter) collected using SCUBA (self-contained underwater breathing apparatus). The subcores were extruded into pressure tubes (Bellco Glass) or 20-ml serum bottles (Wheaton Scientific) under an atmosphere of 93% nitrogen and 7% carbon dioxide. The vessels were stoppered with butyl rubber stoppers (Bellco Glass), sealed with an aluminum crimp, and incubated at in situ temperatures. The rate of increase in methane concentration in the headspace was measured at intervals over a 20- to 30-h incubation period. The tubes were shaken before each methane analysis to equilibrate the dissolved gases with the headspace. Methane was analyzed on a Varian 600D gas chromatograph as described below.

Laboratory studies. Sediments for laboratory studies were collected from the A and B site with an Eckman dredge. Depending on the experiment 500, 700, or 800 ml of sediment was transferred under anaerobic conditions to 1-liter reagent bottles (Wheaton Scientific) and sealed with a rubber stopper.

A final concentration of either 10 or 20 mM ferrous sulfate (sulfate-amended sediments) or ferrous chloride (control sediments) was added to the sediments. Ferrous salts were used to prevent the accumulation of free sulfide, which is toxic to methanogens at high concentrations (7, 29). Ferrous chloride was added to control flasks to eliminate any potential differential effects of excess iron on hydrogen uptake or production. The sediments were incubated at 20 ± 2°C in the dark without mixing or were placed on a cell production bottle roller (Bellco) and slowly turned. Molybdate was added to the sediments as a nitrogen-flushed 0.5 M solution of sodium molybdate to give a final concentration of 5 mM. Molybdate is regarded as an effective and specific inhibitor of sulfate reduction in sediments (20, 21, 25, 26).

Carbon dioxide and methane in the headspace of the bottles were analyzed on a Carle basic gas chromatograph equipped with a microthermistor detector. The gases were separated on a 1-m column of Poropak N (Waters Associates) with a helium carrier at a flow rate of 20 ml/min and an oven temperature of 60°C. When greater sensitivity for methane was desired, a Varian 600D gas chromatograph with a flame ionization detector was used. Gases were separated with a helium carrier on a 1-m column of Poropak N at 50°C. Hydrogen was analyzed on a Varian 3700 gas chromatograph with a thermal conductivity detector. The gases were separated on a 3-m column of Poropak N with nitrogen as the carrier at 15 ml/min and an oven temperature of 35°C. The detection limit was 0.04 pascals. One pascal is approximately equivalent to 9.9×10^{-6} atm and a dissolved hydrogen concentration of 8 nM. The bottles were shaken vigorously before sampling to equilibrate the dissolved gases with the headspace.

Interstitial water for sulfate analysis was collected by centrifugation and analyzed by high-pressure liquid chromatography. Ions were separated at room temperature on a Vydac column (Anspec; 5 × 0.46 cm) with a solvent of 1 mM phthalic acid (pH 5.5) at a flow rate of 2 ml/min. Sulfate was detected with a Wescan conductivity detector (Anspec).

For the kinetic analysis of hydrogen uptake, 4- or 6-ml samples of sediments were dispensed into roll tubes (25 × 142 mm; Bellco). The tubes were flushed with oxygen-free nitrogen before and during the transfer. In experiments where chloroform was added to sediments, a 50- to 75-ml sample of sediment was first transferred to a 120-ml serum bottle. Chloroform was added directly (final concentration, 0.003% [vol/vol]). The sediments were mixed and dispensed into tubes as above. The tubes were incubated with slow rolling on a tube roller to create a thin film of sediment (27). Hydrogen was added, and headspace samples were withdrawn over time and analyzed for hydrogen or methane or both.

Two experimental approaches were used to ensure that chloroform did not alter the potential of SRB to take up hydrogen. In the first experiment, sediments were amended with 550 μM (final concentration) sulfate to saturate SRB for sulfate. The sediments were incubated under saturating hydrogen (50 kPa) on the tube roller, and the rates of sulfate depletion over a 2-h incubation period in sediments treated with chloroform and control sediments were compared. In the second experiment, sediments that had been adapted to 20 mM sulfate were incubated on the tube roller with an initial hydrogen concentration of 1 kPa. The initial rate of hydrogen uptake was measured in untreated sediments, sediments treated with chloroform, and sediments treated with molybdate. If chloroform did not inhibit hydrogen uptake by SRB, then the sum of hydrogen uptake in sediments treated with chloroform and sediments treated with molybdate would equal the hydrogen uptake in untreated sediments.

Kinetic analysis. Hydrogen uptake in sediments has previously been shown to follow Michaelis-Menten kinetics (27).

$$V = \frac{V_M \times S}{K + S} \tag{1}$$

where V is the velocity of uptake, V_M is the maximum potential uptake velocity, S is the substrate concentration, and K is the substrate concentration at which $V = 0.5\ V_M$. Kinetic parameters were estimated from progress curves of hydrogen consumption over time. A linearized expression of an integrated form of the Michaelis-Menten expression can be derived (23).

$$\frac{\ln S_0/S_t}{t} = \frac{-1}{K} \times \frac{S_0 - S_t}{t} + \frac{V_M}{K} \tag{2}$$

where S_0 is the initial substrate concentration and S_t is the substrate concentration at time t. This method gives kinetic parameters for hydrogen uptake in sediments comparable to those estimated from initial velocity studies (27) and has the added advantage that variability between sediment samples for a particular kinetic analysis can be eliminated since all the substrate concentrations are, in effect, tested on the same sediment sample.

Sediments containing hydrogen-consuming MB and SRB populations can be expected to have a total hydrogen uptake described by a two-term Michaelis-Menten equation.

$$V_T = \frac{V_{MSRB} \times S}{K_{SRB} + S} + \frac{V_{MMB} \times S}{K_{MB} + S} \tag{3}$$

TABLE 1. Relative importance of methane production and sulfate reduction in the surface sediments (0 to 2 cm) of Wintergreen Lake during summer stratification

Sediment site	Sulfate concn (μM)	Methane[a] production	Sulfate[a] reduction	Sulfate reduction[b] (% of total)
A[c]	71	40 ± 10	6.2 ± 1.7	13
B	59	26 ± 12	4.0 ± 1.3	13

[a] Micromoles per liter of sediment per hour; mean ± standard error of 3 or more rate measurements.
[b] Sulfate reduction rate divided by total of sulfate reduction rate and methane production rate.
[c] Sulfate concentration and reduction rate for A site from King and Klug (11).

where V_T is the total rate of hydrogen uptake, V_{MSRB} and K_{SRB} are the V_M and K of the SRB population, and V_{MMB} and K_{MB} are the V_M and K for the MB. This two-term equation was used in the analysis of hydrogen uptake in sulfate-containing sediments that had both MB and SRB populations. Kinetic parameters for the two populations were entered into a program which calculated total hydrogen uptake over time.

RESULTS

Concurrent methane production and sulfate reduction were observed in the surface sediments (0 to 2 cm) of both site A and site B (Table 1). Methane production was the dominant process and comprised about the same proportion of the total of methane production and sulfate reduction at both sites.

Methane production in sediments from both sites was completely inhibited within 2 to 5 days at 20°C by the addition of 10 or 20 mM sulfate. Active sulfate reduction in the sulfate-amended sediments was evidenced by the loss of dissolved sulfate and the appearance of black ferrous sulfide over time. There was also an increase in carbon dioxide production in sulfate-amended sediments over that in control sediments.

Sulfate-amended sediments in which methane production was inhibited had significantly lower hydrogen partial pressures than $FeCl_2$ controls and untreated sediments (Table 2). Monitoring over time demonstrated that the inhibition of methane production and the decrease in hydrogen were concurrent (Fig. 1). Both control and sulfate-amended sediments had high initial rates of methane production and elevated hydrogen partial pressures, presumably due to disturbances in carbon flow resulting from the initial manipulations with the sediment. The hydrogen partial pressure stabilized in control ($FeCl_2$-amended) sediments at approximately 1 Pa, whereas methane production continued at lower rates. However, in sulfate-amended sediments the methane production rate and hydrogen partial pressure dropped sharply until methane production was no longer detectable. The hydrogen partial pressure continued to slowly decline after methane production had ceased.

TABLE 2. Hydrogen partial pressure in sediments with and without added sulfate

Sediment treatment	Methane[a] production	Hydrogen partial pressure[b] (Pa)
No additions	+	1.11 ± 0.16
Plus $FeCl_2$[c]	+	1.09 ± 0.14
Plus $FeSO_4$[c]	−	0.17 ± 0.16

[a] +, Indicates detectable methane production; −, indicates methane production was not detectable.
[b] Mean ± standard error of five observations.
[c] Incubated at least five days, but less than 5 weeks, with added $FeCl_2$ or $FeSO_4$.

Addition of 5 mM (final concentration) sodium molybdate to inhibit sulfate reduction in the sulfate-amended sediments resulted in the resumption of methanogenesis at a rate comparable to that in control sediments (Fig. 1). This corresponded with an increase in the hydrogen partial pressure which, after an initial accumulation, stabilized at partial pressures similar to those in control sediments. Molybdate had no effect on the hydrogen partial presure in control sediments (data not shown).

Since MB maintained their potential to metabolize hydrogen in sulfate-amended sediments, a suitable inhibitor that would prevent MB from taking up added hydrogen but would not inhibit hydrogen uptake by SRB had to be found before kinetic analysis of hydrogen uptake by SRB could be made. Chloroform (0.003% [vol/vol]) inhibited methane production but had no significant effect on the potential of SRB to metabolize hydrogen, as measured by the rate of sulfate reduction or the rate of hydrogen uptake (Table 3).

Sulfate-amended sediments had a higher potential for hydrogen uptake than control sediments (Fig. 2, Table 4). The addition of chloroform to the control sediments resulted in the accumulation of hydrogen as previously shown (12), but in sulfate-amended sediments a significant potential for hydrogen uptake remained (Fig. 2, Table 4). The V_M of the population that was inhibited by chloroform in the sulfate-amended sediments can be calculated as the

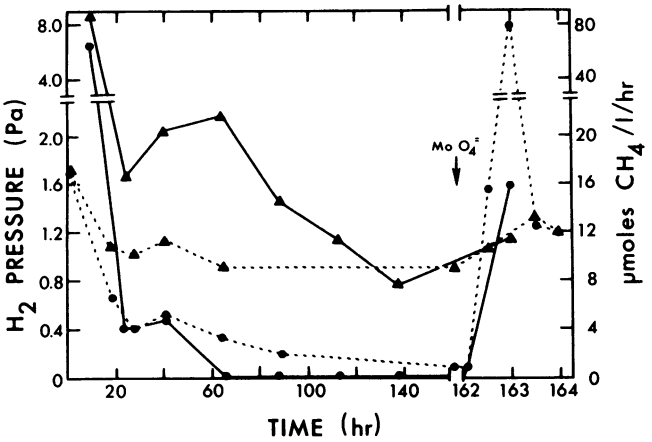

FIG. 1. Methane production rates and hydrogen partial pressures over time in sulfate-amended and control sediments collected from the A site and incubated at 20°C on a bottle roller. Arrow designates addition of molybdate to sulfate-amended sediments. Values are means of duplicate bottles of each treatment and are representative of the results obtained in several similar experiments. Symbols: ●—● and ▲—▲, methane production rates in sulfate-amended and control sediments; ●---● and ▲---▲, hydrogen partial pressure in sulfate-amended and control sediments.

difference between the V_M in the sulfate-amended sediments with and without added chloroform. The value obtained, 0.8 mmol of H_2 per liter of sediment per h, was equivalent to the V_M of the control sediments. This indicates that the hydrogen uptake potential of the MB population was not changed in the sulfate-amended sediment, but that there had been an increase in a hydrogen-consuming potential that was not inhibited by chloroform.

Half-saturation constants, K, for hydrogen uptake were lower in sulfate-amended sediments than in control sediments (Table 4). When the MB in sulfate-amended sediments were inhibited with chloroform, the resultant K was threefold lower than the K in control sediments. When the results of kinetic analyses on sediments collected throughout the summer of 1981 from both the A and B site were compiled, the overall mean K value and 95% confidence interval for hydrogen uptake not inhibited by chloroform was 141 ± 33 Pa (n = 8). This compared with the K for MB in control sediments of 597 ± 186 Pa hydrogen (n = 8).

TABLE 3. Effect of chloroform on the hydrogen uptake potential of methanogens and sulfate reducers

SRB parameter measured	% Inhibition by chloroform[a]	
	Methane production[b]	Sulfate reduction
Sulfate reduction	>94	0.7 (8.9)[b]
Hydrogen uptake	>96	6.1 (9.8)[c]

[a] Mean with standard error in parentheses; n = 3 for each treatment.

[b] Percent inhibition equals (1 − [rate in sediments treated with chloroform × rate in control sediments^{-1}]) × 100. A minimum estimate for methane inhibition is shown since there could have been methane production at rates lower than what could be detected during the incubation period.

[c] Percent inhibition equals (1 − [sum of the rate of hydrogen uptake in sediments treated with chloroform and sediments treated with molybdate × uptake rate in controls^{-1}]) × 100.

The theoretical progress curves of hydrogen uptake in sulfate-amended sediments that were calculated from the two-term Michaelis-Menten expression (equation 3) closely corresponded with those observed experimentally (Fig. 2). For these calculations V_{MSRB} and K_{SRB} were taken as the mean values from the chloroform-treated, sulfate-amended sediment. It was assumed that V_{MMB} was equal to 0.8 mmol of H_2 per liter per hour, as calculated above, and that K_{MB} was equal to the K in control sediments.

DISCUSSION

The fact that the inhibition of sulfate reduction in sulfate-amended sediments resulted in an increase in the hydrogen partial pressure and methane production rates to levels found in methanogenic sediments demonstrated that when sulfate concentrations were not limiting, SRB inhibited methane production by lowering the hydrogen partial pressure below a threshold level necessary for hydrogen utilization by MB. The inhibition of methane production was not due to the toxic presence of sulfate or sulfide, as previously demonstrated (1, 2, 6, 14, 29) nor to the depletion of some factor other than the electron donors necessary for methanogenesis.

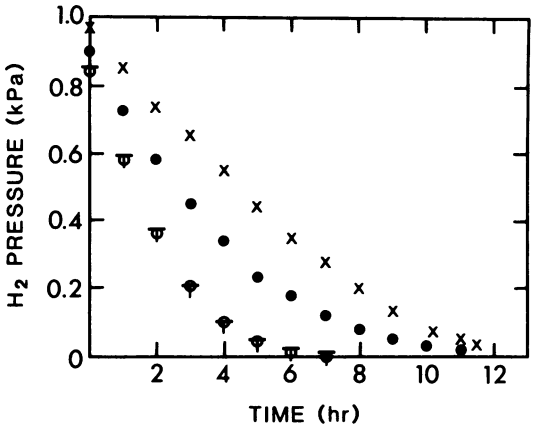

FIG. 2. Typical hydrogen uptake progress curves: Symbols: ○, sulfate, amended sediments; X, sulfate-amended sediments treated with chloroform; ●, control sediments. T represents expected hydrogen partial pressure in sulfate-amended sediments calculated from equation 3 and the appropriate kinetic parameters as described in the text.

This conclusion was further supported by the comparable V_M for hydrogen uptake by MB in control and sulfate-amended sediments. Thus, the inhibition of methane production by added sulfate differs from the inhibition by oxygen (31) or nitrogen oxides (3) where the added electron acceptor or a product of its metabolism directly inhibits MB.

The inhibition of methane production at low hydrogen partial pressures was probably due to the decreased energy yield from methane production. The available free energy for methane production from hydrogen was calculated from the standard free energy of −139.23 kJ (30) and the methane, hydrogen, and carbon dioxide partial pressures to be −16.3 to −16.8 kJ/mol of methane produced in the control sediments shown in Fig. 1. The calculated free energy was approximately −6.7 kJ/mol of methane produced in the sulfate-amended sediments during the initial days of the inhibition of methane production and +4.7 kJ at 6 to 7 days after the sulfate addition. Though care must be taken in extrapolating from bulk-phase pool sizes to those actually experienced by the bacteria, it is clear that the hydrogen partial pressure in the sulfate-amended sediment was sufficiently lowered to significantly reduce the energy available for methane production from hydrogen.

TABLE 4. Kinetic parameters for hydrogen uptake in sediments collected from the A site

Sediment type	Kinetic parameters[a]	
	V_M[b]	K (Pa)
Control sediments	0.8 ± 0.1	588 ± 70
Sulfate-amended	1.2 ± 0.2	455 ± 111
Sulfate-amended treated with chloroform	0.4 ± 0.04	175 ± 45

[a] Mean and standard error of values from triplicate progress curves for each treatment. Progress curves were run concurrently with those shown in Fig. 2.

[b] Millimoles of hydrogen per liter of sediment per hour.

The lower hydrogen pool in the sulfate-amended sediments was associated with the lower overall K for hydrogen uptake and, specifically, with the low K for hydrogen uptake by the bacterial population that was not inhibited by chloroform. The K for hydrogen uptake in chloroform-treated, sulfate-amended sediments is considered to represent the K for the SRB population because: (i) there was no detectable hydrogen uptake in the presence of chloroform in sediments not amended with sulfate; (ii) chloroform did not affect hydrogen uptake by SRB; and (iii) molybdate inhibited the hydrogen uptake in sulfate-amended sediments that chloroform did not inhibit. The K for the MB reported here is within the range estimated independently for methanogenic sediments and other methanogenic environments, such as sludge digestors and the rumen (J. A. Robinson and J. M. Tiedje, submitted for publication). Though there was a possibility of hydrogen uptake by bacteria fermenting hydrogen and carbon dioxide to acetate, the importance of these bacteria in methanogenic environments is low relative to methanogens (5, 13). The conclusion that MB and SRB were the only two important hydrogen-consuming populations is further supported by the observation that the total hydrogen uptake in the sulfate-amended sediments could be predicted by using the K for the sulfate-depleted control sediment as the K for the population inhibited by chloroform.

Under steady-state conditions in environments, such as sediments, where there is negligible physical removal or dilution of the microbial population, the substrate pool size can be described by:

$$S = \frac{K}{(V_M \times y/k) - 1} \quad (4)$$

where y and k are yield and mortality constants and K and V_M are expressed on a per cell basis (4, 15). Thus, the hydrogen partial pressure should be dependent solely upon the physiological characteristics of the hydrogen-consuming populations. In the sulfate-amended sediments, the lower SRB K for hydrogen uptake (and possibly a higher yield and V_M per cell) resulted in a lower hydrogen pool. Some of the inhibition

of methane production in sulfate-amended sediments may be attributed to the metabolism of substrates by SRB rather than proton-reducing bacteria and the subsequent lower rates of hydrogen production (6). However, the maintenance of a lower hydrogen partial pressure by SRB that consumed hydrogen was the ultimate cause of the complete inhibition of methane production since the hydrogen partial pressure was independent of the rate of hydrogen production.

The maximum potential rate of substrate uptake is equally important as the affinity for substrate in determining the outcome of competition (9). The slow inhibition of methane production in Wintergreen Lake sediments amended with 20 mM sulfate can be explained by the small initial potential for hydrogen uptake of SRB. In freshly collected sediments incubated with saturating hydrogen, the turnover time for 1 mM sulfate (a saturating sulfate concentration) is 204 h (25). Assuming that all of the sulfate reduction was due to hydrogen uptake, this yields a maximal V_M estimate for the SRB population of 19.6 μmol of hydrogen per liter of sediment per h. With the estimate that hydrogen is the precursor for approximately 40% of the methane production in these sediments (12), the rate of hydrogen production can be calculated from the methane production rate (Table 1) as 64 μmol per liter of sediment per h, or threefold higher than the SRB V_M for uptake. Using the V_M and K for the MB, the K for the SRB, and the hydrogen partial pressure determined in the present study, it can be calculated from equation 3 that, at saturating sulfate concentrations, SRB would initially be able to use at most only 10% of the total hydrogen consumed by the two populations. Since the in situ sulfate concentration in these sediments is typically at or below the SRB K for sulfate reduction (24), the limitation of SRB by sulfate can be expected to lower the SRB maximum potential for hydrogen uptake (22) and result in an in situ hydrogen uptake by SRB that is much less than 10% of the total hydrogen turnover. This result calculated from kinetic parameters agrees well with previous conclusions derived from experimental results (12).

MB are able to compete successfully with SRB in Wintergreen Lake sediments despite the lower SRB K for hydrogen uptake because the maximal potential for hydrogen uptake by SRB is limited by sulfate availability. The competition between SRB and MB for acetate is expected to have similar mechanisms as those for hydrogen competition. MB and SRB should coexist in other anaerobic sulfate-containing environments in which the rate of sulfate supply supports a potential for hydrogen and acetate uptake by SRB that is lower than the rate of hydrogen and acetate production.

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