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The effect of bituminous coal on methanogenic mixed cultures and pure cultures of *Methanococcus* and *Methanosarcina*

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HIGHLIGHTS

- Bituminous coal was inhibitory to pure cultures of methanogens.
- In general, coal is likely inhibitory to microorganisms.
- Inhibition likely effects the distribution of biogenic methane in coal basins.

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ABSTRACT

In numerous global coal–bearing basins, methane, possessing a biogenic stable isotope composition, is spatially and temporally associated with groundwater recharge. However, beyond groundwater inoculating the subsurface with microorganisms, the biological mechanisms that control the distribution of biogenic methane are poorly understood. In this study, we examined the interactions between bituminous coal and a) methanogenic microbial communities sourced from the goaf of a Bowen Basin underground coal mine and b) pure cultures of methanogens. When coal mine microbial consortium was amended with acetate and a low concentration of coal (1 g in 25 mL medium), methane production was stimulated compared to the addition of acetate alone, though the presence of coal did not affect the methane production from H_2/CO_2. To test whether methanogens benefited directly from the addition of coal, 1 g of either quartz sand or bituminous coal was added to a pure culture of *Methanococcus maripaludis*, a hydrogenotrophic methanogen isolated from the coal mine and grown on H_2/CO_2, and to a pure culture of *Methanosarcina barkeri*, an acetoclastic methanogen grown on acetate. In this experiment coal was not included as an energy source for microbial growth but to test interactions between coal and methanogens. The presence of coal in the medium did not affect methane production by *Ms. barkeri* but slightly inhibited methane production by *M. maripaludis* at the start of the growth phase. Scanning electron microscopy revealed that *M. maripaludis* cells were attached to both sand grains and coal particles, with preferential attachment to rough surfaces, such as cracks within broken coal pieces and clay-rich areas of coal. When the experiment with *M. maripaludis* and *Ms. barkeri* was repeated with a 1:1 volumetric ratio of bituminous coal to medium, which more accurately reflects the environmental conditions of a coal seam, methane production by *M. maripaludis* and *Ms. barkeri* was completely inhibited. In addition, *M. maripaludis* cell numbers declined after inoculation. This suggests that at a high coal: fluid ratio, methanogenesis is inhibited by some component of the coal, *e.g.*, bitumens in the coal or coal-sourced hydrocarbons dissolved into the medium. Based on these laboratory results, we propose that in coal seams, the dilution of inhibitory compounds may be one mechanism by which groundwater recharge promotes biogenic methane production.

1. Introduction

The discovery of coal seam gas with a biogenic stable isotope composition in numerous shallow coal basins has invigorated research on the biodegradation of coal into economically viable products [1–3]. The primary goal of this research has been to increase microbial methane production, either *in situ* in the coal seam so that depleted gas fields may become productive again or in waste coal as an alternate energy source. However, coal has several difficulties for use as a microbial substrate, including a microporous physical structure, a heterogeneous chemical composition

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and the presence of persistent and potentially toxic organic molecules [4].

The production of biogenic methane from coal requires a complete methanogenic consortium. In the community, various groups of microorganisms break down the complex organics in coal into the simpler substrates that are subsequently used by methanogens [1]. Most methanogens, the Archaea that produce methane, are only able to metabolize simple molecules, such as hydrogen, acetate and formate and therefore, are unlikely to directly consume coal (Table 1; [2,5]). An exception is the species *Methermicoccus* that were recently found to be able to metabolize methoxylated aromatic compounds present in coal [6]. In coal seams and mines, 16S rRNA amplicon microbial surveys have found members of *Methanosarcinales*, *Methanobacteriales*, *Methanococcales* and *Methanomicrobiales* [7–12]. Several other studies have also reported methane generation when acetate or $H_{2(g)}$ was added to coal seam microbial communities [13–16] and numerous studies have reported methane generation from coal or coal seam gas water in laboratory cultures (see reviews by Strapoć et al. [1], Meslé et al. [2] and Ritter et al. [3]).

Results from a microbial consortium grown on coal under realistic geochemical conditions suggest that the natural rate of methane production from coal is relatively slow (unpublished work). Several previous studies have proposed the addition of an amendment to coal to encourage coal biodegradation through cofeeding, for example hydrogen or acetate [13,17], tryptone [18,19] and yeast extract [20,21]. In one study by Penner et al. [18], biogenic methane production from a culture medium containing coal and tryptone was 55 times greater than coal alone and 4 times greater than tryptone alone. Adding simple organic carbon substrates into coal seam gas wells to encourage increased methane production has also been tested by several companies [3].

Two hypotheses may explain the increase in methane production when mixed microbial consortia are grown on a combination of coal and an organic carbon supplement. First, coal may provide an additional carbon substrate or inorganic nutrients to the heterotrophic bacteria that increase the release of hydrogen, formate, acetate or methyl-compounds into solution, which are subsequently used by methanogens. Alternatively, the coal might provide something of direct benefit to the methanogens. For example, metals may be present in the coal that could help the methanogens use an available substrate(s). Trace metals, such as nickel, cobalt, iron, selenium and tungsten, are cofactors for methanogenic enzymes and strongly stimulatory in some species [22]. To determine if coal has a positive or negative effect on methanogens, we conducted a series of experiments where bituminous coal was either present or absent in an ideal methanogen medium, i.e., a headspace of H_2/CO_2 (80:20, v/v) for the growth of hydrogenotrophic methanogens or 10–12 mM acetate for the growth of acetoclastic methanogens. Coal was not added as the energy or carbon source for microbial growth but to examine microbe-coal interactions under ideal methanogen growth conditions. In the first experiment, a mixed microbial community from a coal mine goaf, which is the collapsed section of a longwall mine, was grown on methanogenic substrates (H_2/CO_2, acetate, and methanol), with and without the addition of a small amount of bituminous coal. In the second experiment, a pure culture of hydrogenotrophic methanogens grown on H_2/CO_2 and acetoclastic methanogens grown on acetate were cultured in the presence of either coal or silica sand at various water:rock or coal ratios. Understanding microbial interactions with coal in laboratory cultures may help determine what influences, or controls biogenic methane production in coal seams.

Table 1
Energy from methanogenesis [5].

Metabolic process	Reaction	$\Delta Gr^{\circ\prime}$ (kJ/CH_4)
Hydrogenotrophic	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−135
Acetoclastic	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	−31
Methylotrophic	$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H_2O + H^+$	−105

2. Methods

2.1. A coal mine sourced methanogenic mixed microbial community

Microbial consortia experiments were conducted in 50 mL crimp top vials with 25 mL of medium and if applicable, 1 g of medium volatile bituminous coal. Coal was sourced from a Bowen Basin coal mine transfer pile, wet sieved to between 355 and 155 μm and then air dried. The rank of coal from this mine is medium volatile bituminous with a Rv_{max} of 1.3% [23]. The basal salt medium contained (per L), 5 g NaCl, 0.3 g $MgCl_2{\cdot}6H_2O$, 0.3 g NH_4Cl, 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 0.15 g $CaCl_2{\cdot}2H_2O$, 0.02 g KCl, 0.005 g NaF, 10 mL Wolfe trace metal mix (modified according to Meador et al. [24]), 10 mL Wolfe vitamin mix and 0.5 mg resazurin. The medium had a similar total dissolved solids (TDS) concentration as the groundwater from which the microbial consortia was sourced. The medium was reduced by boiling for 10 min, flushing with 100% $N_{2(g)}$ for 10 min prior to addition of 0.5 g L^{-1} $NaHCO_3$ and 1 mL L^{-1} of 0.1 M Na_2S solution. Acetoclastic medium contained 0.820 g L^{-1} of sodium acetate (10 mM acetate) and methylotrophic medium contained 0.4 mL L^{-1} of methanol (10 mM methanol). Medium (pH = 7.5) was dispensed into vials in an anaerobic chamber, the head space was flushed with H_2/CO_2 (80:20) or 100% N_2 and autoclaved.

Water samples were collected by mine personnel in June 2015 from a pipe that drained water from the goaf (decommissioned and collapsed mine section) of a central Queensland coal mine. During collection, 1 L Schott bottles were filled completely, sealed with thick butyl rubber stoppers and immediately shipped to the laboratory. The water pH was 7.4 and this pipe had been previously sampled in April and December 2013 for 16S rRNA microbial survey (samples GOAF-A7 and GOAF-A8 in Raudsepp et al. [12]). The coal mine water contained a cell density of ca. 10^5 cells mL^{-1}, as determined by microscopy. In an anaerobic chamber, 5 mL of coal mine water was inoculated to medium and the cultures were then incubated at 30 °C for 5 weeks. The chemical composition of the inoculum (with added Na_2S) was analysed at the Analytical Services Laboratory at the Advanced Water Management Centre, the University of Queensland. The headspace was not flushed after inoculation and some dissolved methane was released from the goaf mine water at the start of the experiment. In abiotic controls, after goaf mine water was added, the vials were autoclaved at 121 °C for at least 30 min.

2.2. Pure cultures of methanogens

A pure culture of a hydrogenotrophic methanogen was isolated from a coal mine enrichment culture using repeated serial dilutions in the above basal salt medium, H_2/CO_2 (80:20) headspace and 100 units L^{-1} of penicillin and 0.1 mg L^{-1} streptomycin (P4333; Sigma Aldrich). From this pure culture, DNA was extracted using a PowerSoil DNA extraction kit (#12888-100, MoBio CA) and quantified using a Qubit fluorometer and dsDNA BR assay kit (Invitrogen). The 16S rRNA gene was amplified using primers ARC8f (5′-tccggttgatcctgcc-3′) and ARC915r (5′-aaaggaattggcgggg gagcac-3′) in a 50 μL mixture containing 1x PCR buffer (10x buffer stock consists of 200 mM Tris-HCl pH 8.4 and 500 mM KCl), 3 mM MgCl2, 0.2 mM dNTPs, 0.14 μM of primers 27f and 1492r, 1U Plat-