Stimulation of Methane Generation from Nonproductive Coal by Addition of Nutrients or a Microbial Consortium[▽]

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Biogenic formation of methane from coal is of great interest as an underexploited source of clean energy. The goal of some coal bed producers is to extend coal bed methane productivity and to utilize hydrocarbon wastes such as coal slurry to generate new methane. However, the process and factors controlling the process, and thus ways to stimulate it, are poorly understood. Subbituminous coal from a nonproductive well in south Texas was stimulated to produce methane in microcosms when the native population was supplemented with nutrients (biostimulation) or when nutrients and a consortium of bacteria and methanogens enriched from wetland sediment were added (bioaugmentation). The native population enriched by nutrient addition included *Pseudomonas* spp., *Veillonellaceae*, and *Methanosarcina barkeri*. The bioaugmented microcosm generated methane more rapidly and to a higher concentration than the biostimulated microcosm. Dissolved organics, including long-chain fatty acids, single-ring aromatics, and long-chain alkanes accumulated in the first 39 days of the bioaugmented microcosm and were then degraded, accompanied by generation of methane. The bioaugmented microcosm was dominated by *Geobacter* sp., and most of the methane generation was associated with growth of *Methanosaeta concilii*. The ability of the bioaugmentation culture to produce methane from coal intermediates was confirmed in incubations of culture with representative organic compounds. This study indicates that methane production could be stimulated at the nonproductive field site and that low microbial biomass may be limiting *in situ* methane generation. In addition, the microcosm study suggests that the pathway for generating methane from coal involves complex microbial partnerships.

Geopolymers such as coal and shale are significant sources of natural gas (38), a clean-burning energy form. Gas accumulated in coal formations can be collected safely and with minimal disturbance of landforms. Because methane can be produced biogenically, it is theoretically possible to stimulate new methane generation in existing wells and plays. However, geopolymers are composed of recalcitrant organics that are resistant to anaerobic degradation, and they are often associated with environments low in terminal electron acceptors (TEAs) for respiration and nutrients to support microbial activity. More research is needed to understand the mechanism of anaerobic geopolymer degradation and to choose appropriate methods to stimulate new biogenic methane production.

Biogenic activity in geologic formations has been identified, using stable isotope and gas compositions, and correlated with environmental characteristics such as uplift and recharge (14, 30, 48). A number of coal bed and shale studies have included limited microbial surveys (see, for example, references 40, 41, and 45), with an emphasis on methanogens. The precursors of biogenic methane formation are limited to simple compounds, primarily acetate and H₂ (14). The observation of a wide array of solvent-extractable organic compounds in formation groundwaters, including aromatics and long linear, branched, and substituted aliphatics (34), indicates that complex organics may serve as intermediates, and methanogens are unlikely to be the direct agents of coal degradation due to their limited substrate capabilities. The presence of organic inter-

mediates in formation water suggests that the generation of biogenic methane involves a stepwise biodegradation of coal as previously observed for biopolymer degradation (49), and methanogenesis is unlikely to be the rate-limiting step.

In order to gain a better understanding of the factors limiting methane formation, we collected a coal sample from a nonproductive U.S. Geological Survey (USGS) test well drilled in south Texas (Zavala County; 2006). The coal is Tertiary in age, part of the Indio Formation of the Wilcox Group in the Gulf Coast Basin, and is subbituminous in rank. The well is located within an area of large biogenic gas plays (a set of known or postulated oil and/or gas accumulations sharing similar geologic properties) with coal of similar rank and age. Methane recovery from production wells in the Gulf Coast Basin (which covers parts of seven states in the southeastern United States) is similar to that from wells in the Powder River Basin (4, 36). However, commercial coal bed methane production from the Wilcox Group in south Texas has been limited, with only one well sporadically producing methane (46). The USGS test well was drilled near the productive well location. However, gas desorption measurements of the coal core produced little to no gas, indicating little if any methane accumulation (46). Although the lack of significant gas in place could be attributed to site characteristics (e.g., coal bed permeability or the lack of an underlying source of thermogenic gas), factors affecting biogenic gas generation were also considered (coal carbon bioavailability, environmental conditions limiting microbial growth, or absence of a capable microbial population). Initial studies indicated that an undefined fraction of negligible mass (0.25%) of the total carbon in the Texas coal sample (designated TX) was bioavailable (24).

In this study we attempted to stimulate microbial methane

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TABLE 1. Identification of source materials and experimental conditions

Sample or treatment	Description
WBC-2.....	Culture before exposure to coal
TX.....	Untreated coal
Treatment 2.....	Bioaugmentation; microcosm with coal, nutrients
Treatment 3.....	Bioaugmentation; microcosm with coal, nutrients, and WBC-2
Treatment 4.....	BES-inhibited microcosm with coal, nutrients
Treatment 5.....	BES-inhibited microcosm with coal, nutrients, WBC-2
Treatment 6.....	Microcosm with coal, nutrient-free salt solution
Treatment 7.....	Microcosm with coal pretreated with HgCl ₂ , nutrients

generation from the TX coal in two ways: (i) by amendment with inorganic nutrients to stimulate the native microbial population attached to the coal (bioaugmentation) and (ii) by amendment with a microbial consortium of bacteria and methanogens (bioaugmentation). Microcosm experiments were designed to begin to elucidate the role of bacteria in the multistep process of coal degradation to methane.

MATERIALS AND METHODS

Source of coal material. A subbituminous coal sample was collected from a USGS gas exploration test well drilled in the Indio Formation (Paleocene-Eocene Wilcox Group) in Zavala County, TX (the sample came from a 330-m depth below land surface; 29.5°C *in situ*). The USGS test well was located approximately 20 km (12.4 miles) southwest and down dip from a productive well location in southeastern Uvalde County (Kincaid, E. D., number 10A). However, desorption analysis of samples from the USGS test well produced little if any gas (24). The coal sample TX was collected at the drill site as soon as the core reached the surface. Coal to be extracted for DNA was immediately frozen. Coal for the microcosm treatments was placed in airtight stainless steel canisters that were purged with helium and sealed to maintain an anaerobic atmosphere. Coal samples were stored for 5 months prior to the incubation setup.

Microcosm setup. Microcosms were constructed aseptically in an anaerobic chamber with the unfrozen anaerobic coal. Coal was broken into chunks small enough to fit through a 13-mm opening (95% of the chunks measured between 0.2 and 1 cm in any dimension). Approximately 7 g of coal was added to each 120-ml (actual volume) serum bottle. A total of 60 ml of anaerobic bicarbonate-buffered solution (2.5 g/liter NaHCO₃ with a gas phase of 80% N₂-20% CO₂) was added to each treatment. The components of each treatment are summarized in Table 1. For treatment 7 (the killed control), coal TX was treated for 20 h with 10 ml of HgCl₂ solution (200 mg/liter) prior to the addition of the bicarbonate-buffered solution. An anaerobic solution of bromoethane sulfonate (BES; final concentration, 10 mM) was added as a methanogen inhibitor (35) in treatments 4 and 5. Bottles were sealed with a Teflon-coated stopper (West Co., Lionville, PA) and an aluminum crimp. In bioaugmented treatments, a late-log-phase, mixed methanogenic culture, WBC-2 (22), was added to provide approximately 2.8×10^8 cells ml⁻¹. The nutrient solution, defined as the medium, consisted of NaHCO₃ (2.5 g/liter), NH₄Cl (0.5 g/liter), NaH₂PO₄ (0.5 g/liter), and KCl (0.1 g/liter), sparged with N₂/CO₂ (80:20), and a complete suite of trace minerals (including 200 µM SO₄) and vitamins (24) formulated to sustain methanogenic growth (but with coal providing the source of organic carbon and energy). In the nutrient-free solution, NH₄Cl and NaH₂PO₄ were replaced by an equivalent concentration of NaCl. Replicate treatments were incubated (statically, in the dark, at 22°C), and representative bottles (sometimes in duplicate) were sampled destructively over time for chemical and microbial analyses. Resazurin was not included in the microcosm medium due to previously observed inhibitory effects on WBC-2. In order to confirm maintenance of anaerobic conditions during incubation, microcosm medium was subsampled into resazurin solution at the termination of the incubation.

Chemical analyses. Methane was monitored over time in each bottle by removing 0.3 ml of the headspace using a gas-tight syringe and analyzing the sample by gas chromatography (GC) using the methods described in Jones et al. (24). At various time points, microcosms were terminated, and solution (50 ml) for organic analyses was filtered through a precombusted glass fiber filter. Solvent-extractable organics were liquid/liquid extracted using dichloromethane to isolate aliphatic and aromatic hydrocarbons and higher-molecular-weight organic acids. The dichloromethane extracts were analyzed by gas chromatography-mass spectrometry for identification of organic compounds as previously described (34). Volatile free fatty acids (formic, acetic, propionic, and butyric) were analyzed on acidified samples using high-performance liquid chromatography-mass spectrometry as previously described (33).

Molecular analyses. Coal samples analyzed included untreated material frozen in the field and material from a bioaugmented microcosm terminated after 78 days of incubation. DNA was extracted from 0.5 g of coal (aseptically subsampled and ground) using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA), following the protocol for isolation of DNA from stool for pathogen detection provided by the manufacturer. Recovery of DNA from coal was better using the stool kit than with soil kits, phenol-chloroform, and other methods tested and has been previously used to extract coal (45). Yields were insufficient for DNA quantification. There was no indication of PCR inhibition. Due to low yield from the untreated coal sample, whole-genome amplification (Repli-G Midi Kit; Qiagen, Valencia, CA) was applied, following the manufacturer's instructions, prior to analysis of methanogens and sulfate-reducing bacteria. In addition, 2-ml aliquots of culture medium were removed from terminated microcosms after the cultures were shaken and the coal was allowed to briefly settle. These samples represent the nonattached microbial community. Cells suspended in the liquid samples were pelleted and then extracted using a Bio-101 Fast DNA Spin Kit for Soil (MP Biomedicals, Irvine, CA) following the manufacturer's instructions. Samples of the WBC-2 culture were analyzed similarly for comparison.

In order to follow changes in the microbial community during the incubations, PCR amplicons were analyzed by terminal restriction fragment length polymorphism (TRFLP) to get a snapshot of the whole community, including rarer members. DNA extracts were amplified with universal 16S rRNA gene bacterial primers FAM-46f and 519r. The 6-carboxyfluorescein (FAM)-labeled product was digested with MnlI (New England Biolabs, Ipswich, MA). Total peak area in the TRFLP profile was determined by adding the area of all peaks representing fragments greater than 70 bp in length with a threshold area of 50. The contributions of individual peak areas were then calculated as percentages of the total peak area.

PCR amplicons were cloned and sequenced as described in Jones et al. (22). *In silico* digests were performed on the sequence data using MacVector (MacVector, Inc.) in order to predict TRFLP fragment length.

Quantitative PCR (q-PCR) was used as a quantitative measure of group-specific cell concentration using selective primers and conditions (Table 2) with Qiagen kits (Qiagen, Inc., Valencia, CA). A Quantitect SYBR PCR kit was used for all analyses with the exception that a TaqMan assay Quantifast probe kit was used for *Methanosaeta* quantification (50). For each reaction, a 0.6 µM concentration of each primer was used, and samples were analyzed either with a Stratagene MX3000P (Agilent Technologies, La Jolla, CA) or a Bio-Rad Opticon (Bio-Rad, Hercules CA) real-time PCR machine. Fluorescence readings were taken after a 82°C postextension heat step. Plasmids containing the gene of interest were used as quantitation standards and were prepared by cloning PCR products into the pCR2.1 plasmid using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Two or more replicates were analyzed for each DNA sample. Abundances were expressed as the number of gene copies per ml of microcosm solution. Average error between replicate analyses is 15%.

Culture testing on organic substrates. In order to confirm the apparent links between the microbial populations and the degradation of organic compounds in the microcosms, representative compounds (Table 3) were tested in incubations with the culture native to the coal and with WBC-2. The native culture was enriched from the most active duplicate (TX19) by transferring (10%) repeatedly into medium with coal as the sole source of organics. The third transfer of the native enrichment TX19 culture and the WBC-2 culture (transferred directly from stock that had never been exposed to coal) were then transferred into medium containing organic compounds (approximately 100 µM) representing the classes of compounds identified in the treatment 3 coal microcosms. The generation of methane was monitored for up to 250 days.

Nucleotide sequence accession numbers. Partial sequences of the 16S rRNA genes have been deposited in the GenBank database under the following accession numbers: *Geobacter*, HM017148 to HM017154; *Azonexus*, HM017155 to HM017156; *Pelotomaculum*, HM017157 to HM017159; *Synergistetes*, HM017160

TABLE 2. Selective primers and conditions used for PCR

Analysis method(s)	Target group	Target gene	Primers	Annealing temp (°C)	Reference(s)
Cloning/sequencing and TRFLP	Universal <i>Bacteria</i>	16S rRNA gene	46f-519r	56	5, 25
	Universal <i>Archaea</i> Methanogen specific	16S rRNA gene <i>mcrA</i>	671f-1526r mcrAf-mcraR	56 56	5 29
qPCR	<i>Geobacter</i>	16S rRNA gene	Geo494f-Geo825r	53	2, 21
	<i>Methanomicrobiales</i>	16S rRNA gene	Mbiales 471f-Mbiales 754r	59	10
	<i>Methanosarcina</i> spp.	16S rRNA gene	Msarcina 180f-Msarcina 511r	59	10
	<i>Methanosaeta</i> spp.	16S rRNA gene	MST702f-MST1862r TaqMan MST 753f	60	50
	All methanogens (methyl co-reductase gene)	<i>mcrA</i>	mcrAf-mcraR	56	29
	Sulfate reducers (disulfite reductase gene)	<i>dsrB</i>	dsrp2060f-dsr4r	55	17, 44

to HM017161; *Firmicutes*, HM017162 to HM017170; pseudomonads, HM017171 to HM017178; *Hydrogenophaga*, HM017179 to HM017182; and methanogens, HM017183 to HM017190.

RESULTS AND DISCUSSION

Coal microbial community. DNA extracted from the untreated coal was successfully amplified using universal 16S rRNA gene bacterial primers. However, the yield was poor. A bacterial clone library was generated, but DNA yield was insufficient for TRFLP analysis. No clones were produced using universal 16S rRNA gene archaeal or methanogen functional (*mcrA*) primers. No methanogens or sulfate-reducing bacteria were detected using qPCR even after whole-genome amplification. The clone library of the bacterial DNA product consisted mostly of gamma- and betaproteobacteria, and two fermentative phylotypes highly similar to *Weissella confusa* and *Leuconostoc* were observed. The dominant bacteria (43% of the clones) had 99% sequence similarity to both *Acidovorax* and *Hydrogenophaga* in the *Betaproteobacteria*, and 13% of the

clones had 99% sequence similarity to *Acinetobacter* in the *Gammaproteobacteria*. Coal solids collected from the bioaugmented microcosm (treatment 3) after 78 days of incubation yielded no clones using archaeal or *mcrA* primers, but 31 bacterial operational taxonomic units (OTUs) were retrieved. The OTUs from the microcosm solids most closely resembled the DNA of the clone library obtained from the untreated coal but also reflected the community observed in the microcosm liquid, with 26% shared OTUs including *Geobacter*, *Acholeplasma*, *Azonexus*, and *Rhodobacter*. Methanogens were detected in the coal microcosm DNA using qPCR, but these represented less than 0.1% of the number of free-living (unattached) methanogens detected in the microcosm.

Biostimulation of the community native to the coal in microcosms with nutrients added. Methane was generated by the community native to the coal in a microcosm with coal and nutrients but not in microcosms without nutrients (Fig. 1) or in the HgCl₂ control. No methane was observed with the methanogen-inhibitor BES added, indicating that methane was not

TABLE 3. Methane and organic intermediates produced from organic substrates by WBC-2 and the native culture TX19

Class of compound ^a	Starting material	Amt of methane produced in culture (μmoles) ^b		Intermediate(s) observed
		TX19	WBC-2	
LCFA (C ₁₈)	Octadecanoic acid	— ^b	532	Hexadecanoic, tetradecanoic, acetate Butyrate, acetate, formate
LCFA (C ₁₆)	Hexadecanoic acid	—	591	
MCFA (C ₁₂)	Dodecanoic acid	—	—	Phenol, cyclohexadiene, methylcyclohexane 1,2-Benzenediol
MCFA (C ₁₀)	Decanoic acid	—	—	
Aromatic	Phenol	—	—	
Aromatic	Benzoate	—	2,953	
Aromatic	Vanillic	53	1,514	ND
Aromatic	Caffeic	—	—	
Alkane (C ₁₆)	Hexadecane	—	—	
Alkane (C ₂₀)	Eicosane	—	1	
Alkane (C ₂₂)	Docosane	ND	91 ^c	ND
Alkane (C ₂₄)	Tetracosane	ND	107 ^c	
PAH	Phenanthrene	ND	—	ND
PAH	Anthracene	ND	—	
PAH	Phenanthrene-quinone	ND	—	

^a MCFA, midchain fatty acid.

^b Values represent methane produced from a 100 μM concentration in the starting material after 130 days unless otherwise noted. —, methane less than or equal to a no-substrate control; ND, not done.

^c Amount of methane produced after 250 days.

FIG. 1. Methane generated from coal by native populations. Data are for treatment 2 with nutrients (TX19 and TX20) and for controls with no nutrients (treatment 6) and with BES added (treatment 4). Methane was produced when TX19 was transferred into new medium with coal on day 105. The total number of methanogens (methyl co-reductase A copies per ml) in TX19 was determined using qPCR. For comparison, TX20 contained 600 *mcrA* copies per ml on day 105. (Inset) natural log of duplicates TX19 and TX20 showing two-phase methane generation, with divergence of the two microcosms during the second phase.

desorbing from the coal. When the enriched microbial population of TX19 was transferred to new medium with fresh anaerobic TX coal, methane generation ensued immediately, again generating $60 \mu\text{mol g}^{-1}$ of coal (Fig. 1). This pattern of methane generation was sustained over four transfers to new coal/medium.

There was a two-phase logarithmic increase in methane (Fig. 1, inset), with only one of duplicate treatments generating methane between 70 and 99 days. This late-stage methanogenesis was accompanied by an increase in the number of *mcrA* copies (Fig. 1). The methanogen clones from TX19 (day 105) were identified by cloning and sequencing as 98% similar to *Methanosarcina barkeri*. *Methanosarcina* is a facultative acetate-clast (capable of using a wide variety of substrates including acetate and H_2). The occurrence of phase 2 methane generation was not linked to substrate availability. Acetate ($383 \mu\text{M}$) in the microcosm bottle that did not generate methane was well above the substrate threshold for methanogenesis. The explanation for the variability between duplicates is not clear but may be related to a critical threshold in a methanogen biomass or an unknown factor limiting (TX20) or supporting (TX19) methanogen growth. Coal material frozen at the field site for molecular analysis yielded no detectable methanogen DNA even after whole-genome amplification, indicating that low biomass certainly could be a factor. Although the nutrient-stimulated native bacterial population differed from that of the negative controls (Fig. 2A and B), there was no discernible difference between the divergent duplicates (TX19 and TX20) with respect to the two bacterial populations (data not shown). This suggests that the dominant bacteria enriched in micro-

cosms with coal and nutrients added played a role in methane production in both TX19 and TX20 but cannot explain the late-phase production of methane in TX19.

It was not possible to determine whether the occurrence of methane production by native organisms was linked to the upstream release of solvent-extractable organics from coal. Coal subsamples used in the incubation of native organisms behaved variably, probably due to heterogeneity of both native organisms and the coal structure. The analysis of organics required microcosm termination, and it was not possible to predict *a priori* which bottles would produce methane at a later step. Addition of the microbial culture WBC-2 resulted in more consistent and reliable replication of methane generation. Therefore, we were able to sacrifice replicate bottles over time and analyze the temporal link between changes in the pool (release and degradation) of solvent-extractable organics, changes in composition of the microbial community, and the production of methane.

Bioaugmentation with WBC-2. Bioaugmentation resulted in more rapid and consistent generation of methane from coal (Fig. 3A). When WBC-2 was added to the microcosm, methane increased in all replicates at a greater rate than with the community native to the coal alone. As observed in the native microcosm, there appeared to be at least two distinct periods of methane generation: a low rate (ca. $0.1 \mu\text{mol methane day}^{-1} \text{g}^{-1}$ of coal) during the first 50 days of incubation and a higher rate (ca. $3 \mu\text{mol methane day}^{-1} \text{g}^{-1}$ of coal) between 50 and 70 days of incubation. Methanogens grew in the microcosms, and most of the growth occurred later in the incubation (Fig. 3B). All of the OTUs in the archaeal clone libraries (39

FIG. 2. Microbial populations in negative controls, source materials, and bioaugmented treatments. TRFLP OTU peak areas (in percents) relative to the sum of TRFLP peak areas are shown. (A) Treatments that did not produce methane. (B) Potential source populations WBC-2 and the native population of treatment 2 (TX19 and TX20 profiles were highly similar; TX19 is shown). (C) Treatment 3, bioaugmented with WBC-2, at two time points (39 and 78 days) during incubation.

days and 78 days) were 98% similar to *Methanosaeta concilii*, an obligate acetoclast. However, the *Methanomicrobiales* group (nonacetoclastic) and *Methanosarcina* (facultative acetoclasts) were also detected in the microcosms using qPCR. The concentration of the *Methanomicrobiales* group was highest early in the incubation and then dropped, and *Methanosaeta* and *Methanosarcina* were lowest early in the incubation and then peaked together at 78 days (Fig. 4). Methane generation was completed by approximately day 70, and the total amount of methane produced was 25% higher than that produced in the

native microcosm (a total of about 80 $\mu\text{mol g}^{-1}$ of coal, or 56 standard cubic feet [scf]/ton). At this point, although the population of methanogens was high and conditions remained anaerobic, it appears that organic precursors were expended (33), precluding further methanogenesis, and the remaining coal carbon was not biodegradable without further modification (24).

Samples were collected at several intervals to characterize the solvent-extractable organic compounds released to solution in the bioaugmented microcosms (treatment 3). When

FIG. 3. Methane and acetate accumulation and the concentration of methanogens in treatment 3 bioaugmented microcosms. (A) Methane generation in individual microcosms prior to destructive termination after 39, 56, 78, and, 102 days of incubation and acetate concentrations after destructive termination at 8, 18, 25, 39, 56, 78, and 102 days. (B) Total methanogens as *mcrA* copies/ml determined by qPCR in bioaugmented microcosms after termination at 8, 18, 25, 39, 56, 78, and 102 days.

coal was added to medium (time zero), traces of fatty acids dodecanoic acid (C_{12}), tetradecanoic acid (C_{14}), and hexadecanoic acid (C_{16}) were immediately detected. Eight days after WBC-2 was added to coal, hexadecanoic acid had increased by about 30 times, and octadecanoic (C_{18}) acid was also present. Long-chain alkanes (C_{22} to C_{36}), phenols, phthalates, and sterol precursors typical of plant material (such as squalene, stigmasterol, and sitosterol) were also present in solution. These signature peaks were gone by day 78 (33). No polyaromatic hydrocarbons were detected in the microcosm solutions. Acetate and traces of propionate and butyrate were detected. The decrease and depletion in acetate corresponded with the cessation in methane production (Fig. 3). The accumulation of acetate in the BES treatments (61 and $78 \mu\text{mol g}^{-1}$ of coal in treatments 4 and 5, respectively) corresponded to the methane generated in the absence of BES. Acetate also accumulated ($29 \pm 2 \mu\text{mol/g}$ coal after 102 days) in treatment 7. WBC-2 demonstrated a capability for degrading representative organic compounds, including long-chain fatty acids (LCFA) and single-ring aromatics and, to a lesser extent, long-chain alkanes (Table 3). TX19 produced a small amount of methane from vanillic acid (not enough to indicate ring cleavage) but did not produce methane from any of the other compounds tested. However, it should be noted that only cells suspended in the medium (not cells attached to the coal) were transferred into substrate tests.

The bacterial community in treatment 3 bioaugmented with WBC-2 had a TRFLP profile completely distinct from the

FIG. 4. Methanogen population changes during incubation of treatment 3. DNA copies/ml using qPCR with 16S rRNA gene selective primers: nonaceticlastic *Methanomicrobiales* group (A), obligate aceticlast *Methanosaeta* (B), and facultative aceticlast *Methanosarcina* (C) in microcosms destructively sampled over time. Note that *Methanosaeta* values are plotted on log scale to depict the full range.

profile of the enriched native community stimulated with nutrients (treatment 2). The dominant TRFLP peaks in the bioaugmented microcosms (Fig. 2C) included two dominant peaks (169 and 254 bp), which were not evident in the source materials (Fig. 2B). A total of 25 phylotypes identified in the bioaugmented microcosm represented bacteria that were not observed in the library of native organisms or in the WBC-2 library (including seven that were found only in the solid coal extraction). These bacteria were apparently too rare to be detected in the source materials but were selectively enriched in the presence of coal and WBC-2. Bacteria similar to those in WBC-2 and the clone libraries of native organisms were also detected in the bioaugmented microcosm. *Pelotomaculum* and two *Bacteroides* OTUs appeared in both WBC-2 and the treatment 3 microcosm clone libraries, and the native bacteria

FIG. 5. Changes in selected bacterial populations during incubation of treatment 3 microcosms. DNA copies/ml using qPCR with 16S rRNA gene *Geobacter* primers (A) and the *dsrB* functional gene for sulfate-reducing bacteria (SRB) (B) in microcosms destructively sampled over time. nd, analysis not done.

(*Pseudomonas* and *Veillonellaceae*) were also detected in the treatment 3 population.

TRFLP was used to examine shifts in the microbial community during the course of the incubations. The peak at 254 bp increased in relative importance from undetectable at 8 days to 64% of the total peak area on day 39. The fragment size correlated well with the *in silico* digest (257 bp) of a *Geobacter* OTU (Dp257; 97% sequence similarity to *Geobacter metallireducens*), which accounted for 67% of the OTUs in the 39-day clone library. *Geobacter* (Dp257) accounted for 10% of the TRFLP area and 11% of the OTUs in analyses of the day 78 microcosm. *Geobacter* abundance tracked using qPCR also peaked at 39 days of incubation and then declined (Fig. 5). Another peak enriched in the treatment 3 microcosm was 169 bp (Fig. 2C). This corresponded to an OTU (Bp171) representing 38% of the OTUs in the 78-day clone library and an *in silico* cut site of 171 bp with 97% sequence similarity to *Azonexus caeni* in the *Betaproteobacteria*. The relative importance of *Azonexus* Bp171, monitored using TRFLP, increased steadily, peaking at 36% of the bacterial population at day 78 of incubation. *In silico* cuts of the *Pelotomaculum* sequence indicate that it cannot be detected using TRFLP with MnlI, and an alternative restriction enzyme for the 46f-519r region could not be identified. However, *Pelotomaculum* did not exceed 5% of the total OTUs in treatment 3 clone libraries.

Microbes and mechanisms. The process of forming biogenic methane from coal can be divided into three events: (i) the release of soluble organic intermediates from the coal geopoly-

mer, (ii) the degradation of soluble intermediates into substrates utilizable by methanogens, and (iii) methanogenesis. Although the degradation of biopolymers such as cellulose is well understood (49), the anaerobic degradation of geologically altered (coalified) organics is relatively less well known. In a model presented by Strapoć et al. (41), the first event is characterized by fragmentation of the coal into small polyaromatic hydrocarbons (PAH) and ketones, in addition to smaller compounds (succinate, volatile fatty acids, CO₂, and H₂) released by fermentation and alkanes released from oil inclusions. In the TX microcosms, we observed the accumulation of acetate to concentrations well above the threshold for methanogenesis as early as day 8 day of incubation, suggesting that acetate was among the first organic compounds released from the coal, presumably by fermentation. However, in contrast with the Strapoć model, we did not observe PAH or ketones in the microcosm solution. Instead, we observed single-ring aromatics, long-chain alkanes, and long-chain fatty acids that accumulated during the first 39 days and were then degraded (33).

The degradation of LCFA (19, 20), aromatics (12, 47), and alkanes (1, 51) has been observed in methanogenic consortia. The degradation of these compounds in the absence of a terminal electron acceptor (TEA) for respiration involves the syntrophic cooperation of microorganisms; i.e., the reactions are thermodynamically driven by the removal of H₂ (32) and result in accumulation of acetate and propionate (Fig. 6). Putative syntrophic bacteria (both *Firmicutes* and *Deltaproteobacteria*) have been previously observed in coal beds and formation waters (40), in oil fields (8, 18), and in coal tar waste waters (3). The syntrophic bacterium *Pelotomaculum* was identified both in WBC-2 and in the bioaugmented coal microcosms. *Pelotomaculum* is known to oxidize phthalates and benzoate (37) and propionate (9) in coculture with a hydrogen-consuming methanogen. H₂-utilizing methanogens were present in the coal microcosms although no growth was observed.

The growth of *Geobacter* Dp257, with cells both suspended in the microcosm fluid and associated with the coal solid, was temporally linked with the increase in soluble organic intermediates, and its numbers remained high as intermediates were degraded. *Geobacter* species are known to degrade aromatics (27, 39) and long-chain fatty acids (6) coupled to reduction of Fe(III) as a TEA. However, none of the TEAs known to support *Geobacter* metabolism were present in the microcosm. There is evidence, however, that some *Geobacter* species can oxidize organics in syntrophic association with other bacteria (7, 42). *Geobacter metallireducens* is genetically similar to *Syntrophus*, which can degrade a wide range of organics with a methanogenic partner (31). The dominance of Dp257 in the coal microcosms suggests that *Geobacter* may also be capable of coupling the degradation of recalcitrant organics to an electron- or H₂-accepting syntrophic partner.

Although microcosm bottles were terminated over time to represent temporal changes, the bottles also reflected variation among replicates. The bottle terminated on day 56 appears to be an outlier. Biomass in this microcosm was low, and complex organics were high (33). Acetate was low, and, correspondingly, methane generation had slowed. Thus, in the absence of a significant *Geobacter* population (Fig. 5), organics accumulated but were not degraded, suggesting that *Geobacter* is as-

FIG. 6. Proposed model of biogenic methane generation from coal based on the current study and previous work in other laboratories (9, 11, 12, 15, 19, 20, 37, 51). H_2 may be removed by methanogenesis, acetogenesis, or by a bacterial partner with a TEA. Mid-chain fatty acids were not observed during the microcosm incubation. (Reference numbers are shown in parentheses.)

sociated with the second event, intermediate degradation. The solubilization of intermediates from coal may be associated with fermentative bacteria (9 of the 10 phylotypes on day 39 were *Firmicutes* or *Bacteroides*). In addition, attached bacteria native to the coal (such as *Hydrogenophaga* and *Acinetobacter*) may have played a role. *Hydrogenophaga* was dominant in coal from the Zavala site and has also been observed in lignite (16). These bacteria are also common in waste treatment reactors, systems which are similarly rich in organics and poor in TEAs. Aromatic oxidation has been observed with oxygen as the TEA in *Hydrogenophaga* and *Pseudomonas* spp. (13), and *Acinetobacter* has been shown to oxidize long-chain alkanes (15). However, the pure cultures required the presence of oxygen or nitrate, TEAs that were not available in the microcosm.

The other dominant OTU in the bioaugmented microcosm was *Azonexus* sp. (formerly *Azoarcus*), a betaproteobacterium that can respire oxygen and nitrate. Although it is not from a recognized syntrophic group, *Azoarcus* was codominant in a coal tar waste-contaminated aquifer, along with syntrophic bacteria (3). In that system, *Azoarcus* was presumed to be using nitrate as a TEA. As the TX coal microcosm did not contain nitrate but did contain organic compounds similar to the coal tar site, it should be considered that *Azonexus* might also have syntrophic capabilities.

To date, syntrophic bacteria have been identified only in two phylogenetic groups of the *Bacteria*: the *Firmicutes* and the *Deltaproteobacteria*. The observation of respiratory bacteria from other phylogenetic groups in coal and coal microcosms (*Beta*- and *Gammaproteobacteria*) may indicate a broader phylogenetic involvement in syntrophic metabolism as a means of electron transfer in environments that are organically rich and TEA poor. Future studies to trace specific degradation pathways could employ methods such as stable isotope probing (28) to confirm which bacteria are involved.

The third event in the coal methanogenesis process is the generation of methane, which can be formed from $H_2 + CO_2$, acetate, methanol, formate, methylamines, or methylated sulfur. Acetate accounted for most of the methane generated in the microcosms. Both acetoclastic and hydrogenotrophic (CO_2 reducing) methanogens were detected in bioaugmented microcosms, but most of the methane production was associated with growth of the obligate acetoclast *Methanosaeta*. Growth of acetoclastic methanogens was not observed until day 56 even though acetate exceeded the threshold for methanogenesis by day 8 in the microcosm.

One of the environmental factors proposed to control methanogenesis in coal is sulfate as sulfate reducers can out-compete methanogens for some substrates (26). Formation water at the Zavala County, TX, site had a moderate concentration of sulfate (approximately 1 mM) although no sulfate reducers were detected in the coal. The microcosm medium included 0.2 mM sulfate, and sulfate reducers were introduced with WBC-2. Sulfate-reducing bacteria were monitored using qPCR with primers selective for the disulfite reductase gene (*dsrB*). Although sulfate reducers (*Desulfovibrio* and *Desulfobulbus*) were present in WBC-2 and *dsrB* was detected at time points throughout the treatment 3 microcosm, no increase was observed until day 78 (Fig. 5B). Therefore, the low rate of methanogenesis early in the incubation cannot be accounted for by sulfate reducers outcompeting methanogens for the available substrates.

Methanogen growth and activity may have been inhibited by the acute toxicity of LCFA (20) or aromatics (37, 43). Hydrogenotrophic cell numbers declined between day 8 and day 39 as organics in solution increased. Once the dissolved organic concentration diminished, rapid methane generation was observed, accompanied by growth of both acetoclastic methanogen populations. Fry and coworkers (16) observed a similar dearth of methanogenic activity within lignite-rich beds, whereas activity was higher in adjacent layers with low total organic carbon, particularly near the lignite interface. This suggests that environmental factors such as organic toxicity may influence the rate, and possibly the pathway, of methane generation from coal. Acetate could serve as a reservoir of methane precursors (via acetogenesis) until the toxic organics are depleted (closed system) or diffuse to a zone of lower carbon (open system). Toxicity could be considered an artifact of the microcosms, but it also has implications for *in situ* treatments in which the release of organics from the coal is accelerated (23).

Summary and implications. The coal from the USGS test well in Zavala County, TX, did not appear to be generating methane *in situ*. The microcosm study demonstrated that it was possible to stimulate the native microbial population to pro-

duce methane from coal by adding nutrients and to enhance the rate and maximum yield of methane production by bioaugmentation with a microbial consortium (WBC-2). Aceticlastic methanogenesis accounted for most of the methane produced in the stimulated treatments.

The pathway by which coal is anaerobically converted to methane is both biochemically and microbially complex. Developing strategies to stimulate coal bed methane would need to include consideration of confounding factors. For example, syntrophic and fermentative bacteria, which appear to be likely contributors to the process, survive near the thermodynamic limits of life (11, 32), and, therefore, their growth is slow. Introduction of electron donors or acceptors, which could stimulate microbial growth, is likely to divert electrons away from methanogenesis. Stimulation of more rapid organic release could result in toxic conditions that could limit biogenic methane generation within the coal bed. Cultivation of highly adapted cultures for use in bioaugmentation may be a practical approach, but successful introduction and maintenance *in situ* will be a challenge. New engineering approaches, including physical alteration of the coal beds, to maintain optimal conditions for microbial activity will likely emerge as the potential pathways, and the roles and limits of specific bacteria and methanogens will be better defined.

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Trade names used in this report are presented for full disclosure of analytical methods. No endorsement of products by the USGS is implied.

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