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microbial biotechnology

Nutrient and acetate amendment leads to acetoclastic methane production and microbial community change in a non-producing Australian coal well

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Summary

Coal mining is responsible for 11% of total anthropogenic methane emission thereby contributing considerably to climate change. Attempts to harvest coalbed methane for energy production are challenged by relatively low methane concentrations. In this study, we investigated whether nutrient and acetate amendment of a non-producing sub-bituminous coal well could transform the system to a methane source. We tracked cell counts, methane production, acetate concentration and geochemical parameters for 25 months in one amended and one unamended coal well in Australia. Additionally, the microbial community was analysed with 16S rRNA gene amplicon sequencing at 17 and 25 months after amendment and complemented by metagenome sequencing at 25 months. We found that cell numbers increased rapidly from 3.0×10^4 cells ml^{-1} to 9.9×10^7 in the first 7 months after amendment. However, acetate depletion with concomitant methane production started only after 12–19 months. The microbial community was dominated by complex organic compound degraders (*Anaerolineaceae*, *Rhodocyclaceae* and *Geobacter* spp.), acetoclastic methanogens (*Methanothrix* spp.) and fungi (Agaricomycetes). Even though the microbial community had the functional potential to convert coal to methane, we observed no indication that coal was actually converted within the time frame of the study. Our results suggest that even though nutrient and acetate amendment stimulated relevant microbial species, it is not a sustainable way to transform non-producing coal wells into bioenergy factories.

Introduction

Coal is the most important fossil fuel on the planet, comprising 70% of the total fossil fuel stock (Iram *et al.*, 2017). Coal mining has a big environmental impact and is estimated to result in 24–42 Tg of methane emissions per year. Methane in coalbeds is produced biologically or thermogenically and escapes from both active and abandoned coal mines. This amounts to 11% of total yearly anthropogenic methane emissions (Saunois *et al.*, 2016) and thus has a considerable climate impact due to the high global warming potential of methane (34 for 100 years, (Myhre *et al.*, 2013)). Coalbed methane can be used as a more environmentally sustainable alternative to direct coal burning (Fakoussa and Hofrichter, 1999; Al-Jubori *et al.*, 2009; Kinnon *et al.*, 2010) as direct coal burning leads to pollution in the form of heavy metals and sulfur compounds (Querol *et al.*, 1995). At the same time, the exploitation of coalbed methane could effectively reduce methane leaking into the atmosphere. In many cases, coalbed methane harvesting is not profitable due to unfavourable system properties, like highest rank coal species or unfeasible methane production rates as well as barriers to a selling market (Moore, 2012). As a result, many active methane-producing coal systems are neglected and thus remain sources of methane emission.

Biogenic coalbed methane production requires a consortium of anaerobes that convert coal compounds to substrates for methanogenic archaea (Jones *et al.*, 2010). Recently, Mayumi and co-workers showed that coal-derived methoxylated compounds can be directly used for methanogenesis by *Methermicoccus* species (Mayumi *et al.*, 2016). Coal biosolubilization, the rate limiting step, is carried out by fungi expressing peroxidases, laccases, hydrolytic esterases and also abiotically by alkaline metabolites and naturally occurring chelators (Fakoussa and Hofrichter, 1999; Strąpoć *et al.*, 2008; Papendick *et al.*, 2011). The microbial degradation of intermediates to volatile fatty acids (VFAs) and the subsequent production of acetate and H_2/CO_2 fuels the methanogens (Robbins *et al.*, 2016).

In Australia, roughly 5000 coal seam gas wells were operated in 2014 (Day *et al.*, 2014) which accounted for 18% of the total gas production in 2014–2015 and almost half of the total East Coast gas production (Ball *et al.*, 2016). Several studies focused on enhancing methane production by applying nutrient, trace element and biotic amendment to low-emitting coal seams (Jones *et al.*, 2010; Penner *et al.*, 2010; Ünal *et al.*, 2012). In Australia, coalbed exploitation for biogas production has gained more attention in the last decade and several studies focused on characterizing and enriching microorganisms from coal (Faiz and Hendry, 2006; Midgley *et al.*, 2010; Papendick *et al.*, 2011; Robbins *et al.*, 2016). However, detailed data on the effects of nutrient amendments on microbial communities in these systems are scarce but highly needed to successfully optimize coalbed methane production.

In this study, we analysed methane production, cell counts, physicochemical properties of the well water and acetate degradation in a sub-bituminous coal well over the course of 25 months. We compared this data to the microbial community composition at 17 months when acetate consumption and methane production were observed and at 25 months, the final time point, using 16S rRNA gene amplicon sequencing. For the 25 month sample, the functional potential of the microorganisms was investigated using a metagenome data set. This data set was also interrogated to study the fungal, archaeal and bacterial communities using ribosomal rRNA gene based phylogeny. By combining this set of complementary experiments, we could reconstruct the complex microbial food web and assess the suitability of nutrient and acetate amendment to transform non-producing coal seams into gas production sites.

Results and discussion

The two experimental coal wells are located at the Lithgow State Coal Mine in the Western Coal Fields of New South Wales, Australia. The coal bearing layers are located 80 m below ground level. About 2 m^2 of coal is continuously exposed to the groundwater that has a volume of 450 L, a pH of 7.9, an oxidation reduction potential of -232 mV (chemical potential relative to the standard hydrogen electrode (SHE)) and a temperature of 16°C. Groundwater flows were not assessed here but they likely cause exchange and mixing of the water body. After 25 months of operation, the pH was 8.2 and the redox potential -197 mV. The headspace gas volume is circa 1000 L. The well was nutrient amended by adding a final concentration of 1.8 mM ammonium and 1.9 mM phosphate in addition to 20 mM acetate in two 10 mM additions at 0 and 4 months. Nutrient and acetate concentrations were pre-assigned according to preliminary *in vitro* culture assays performed and modified according to Jones *et al.* (2010). Nutrient and acetate solutions were slowly released by drop tubing to 450 L coal formation water in contact with the coal seam with a feeding rate of about 0.5 L min^{-1} for a 2 L stock solution.

Amendment stimulated microbial growth and methane production

The amended well showed considerable methane production after 12 months, whereas in the unamended well, no methane production was observed over the course of the whole experiment (25 months, Fig. 1A). These observations are in line with previous studies on microbial coal conversion that showed that nutrient amendment stimulated methane production from non-producing coal systems (Laborda *et al.*, 1997; Jones *et al.*, 2010; Penner *et al.*, 2010). Interestingly, methane emission was only observed after a prolonged lag phase of 12 months in conjunction with acetate degradation and stopped when acetate was depleted after about 18 months. This indicates that the microbial community at the start of the experiment was not yet able to or not abundant enough to convert acetate to methane. When we analysed the cell numbers in the two coal wells, we found that there was considerable growth in the amended well in the beginning of the incubation (3.0×10^4 cells ml^{-1} at 0 months; 9.9×10^7 cells ml^{-1} at 7 months, Fig. 1B) with a stabilization of the cell density after 7 months (2.4×10^8 cells ml^{-1} at 25 months) and absence of growth in the control well (1.5×10^4 cells ml^{-1} at 0 months; 1.6×10^4 cells ml^{-1} at 25 months, Fig. 1B). The increase in cell numbers was not correlated with a decrease in acetate concentration or concomitant methane production so it was presumably mainly due to the addition of nutrients that were also limiting in the unamended coal well. After about 11 months, acetate consumption and methane

production were first observed, indicating a shift in microbial community functioning. From this, it can be concluded that acetate and nutrient amendment clearly stimulated microbial growth and enabled the microbial community to eventually convert acetate to methane. As methane production stopped after acetate depletion, these data also suggest that the methane-producing microbial community was not able to thrive without external addition of acetate (Fig. 1A) even though the microbial biomass had increased by several orders of magnitude (Fig. 1B). Stoichiometric analysis indicated that about 25% of the acetate was converted to methane, suggesting presence of syntrophic acetate oxidation, which has been previously observed in organic carbon-rich petroleum reservoirs (Mayumi *et al.*, 2011). Sulfate-dependent acetate oxidation was unlikely to occur due to the rapid depletion of sulfate from 1 mM at the start of the experiment (Table S1) to below the detection limit after 3 months (< 0.02 mM). The decrease in methane after 19 months in the amended coal wells is probably due to escape of methane from the coal well rather than active degradation of methane. We analysed the sequencing data for aerobic or anaerobic methanotrophs but did not find any indication (16S rRNA gene and marker genes *pmoA*, *mcrA*) of aerobic or anaerobic methane oxidation.

To get insight into the functioning of the microbial food web and as indication as to why methane production stopped after 15 months, we sampled the coal well water at 17 and at 25 months to analyse the microbial communities and their functional potential. Phylogenetic analyses were performed on both 16S and 18S rRNA genes and fungal ITS (Fig. 2, Table S2). Functional analyses targeted genes encoding proteins involved in complex organic compound degradation, metal reduction, electron transfer reactions and volatile fatty acid metabolism (Table S2).

The fungal community has the potential for coal biosolubilization

Fungi represented 15% of the combined 16S/18S rRNA gene reads detected in the metagenome data set (Fig. 2). The sequences were most related to species within the Agaricomycetes class that contains well-characterized wood-rotting fungi (Ohm *et al.*, 2014). Additional identification based on fungal ITS sequences supported identification up to the Basidiomycota division, and most reads (89%) were closely related to *Basidiomycota* sp. MEL 2363319 (KP311433) (99% nt identity). In the metagenome, we found functional genes encoding lignin, manganese and versatile peroxidases that are involved in complex organic compound degradation. Even though these enzymes require oxygen as co-substrate, Agaricomycetes have been previously detected in several anoxic environments such as marine
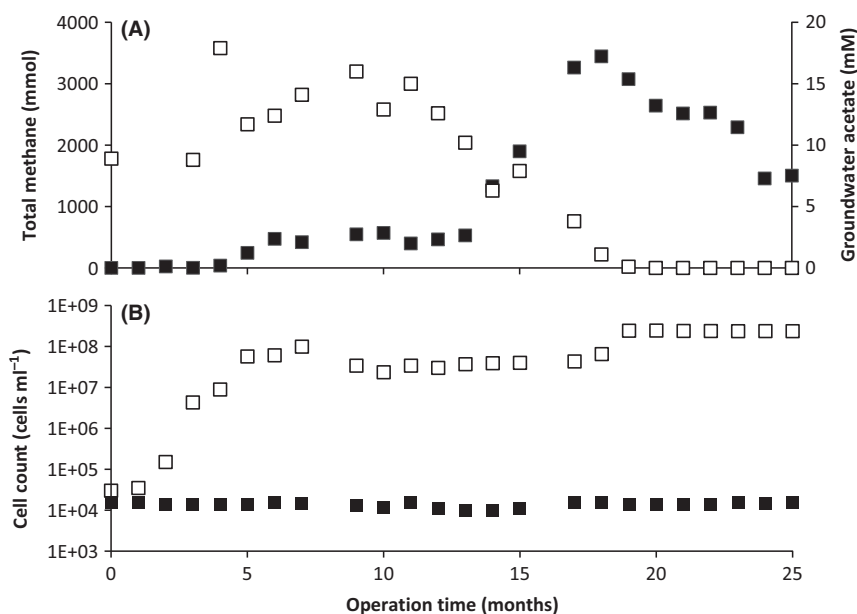

Fig. 1. Monthly methane and acetate concentration measurements in the amended coal well (A) and total cell count data for both the nutrient/acetate amended and the control coal well (B). No methane production was observed in the control coal well. To the amended coal well, 10 mM acetate was added at 0 and 4 months respectively.
A: open squares (□), acetate concentration in mM; filled squares (■), total methane in mmol;
B: open squares (□), total cell count in cells ml^{-1} for the nutrient/acetate amended well; filled squares (■), total cell count in cells ml^{-1} for the control well.
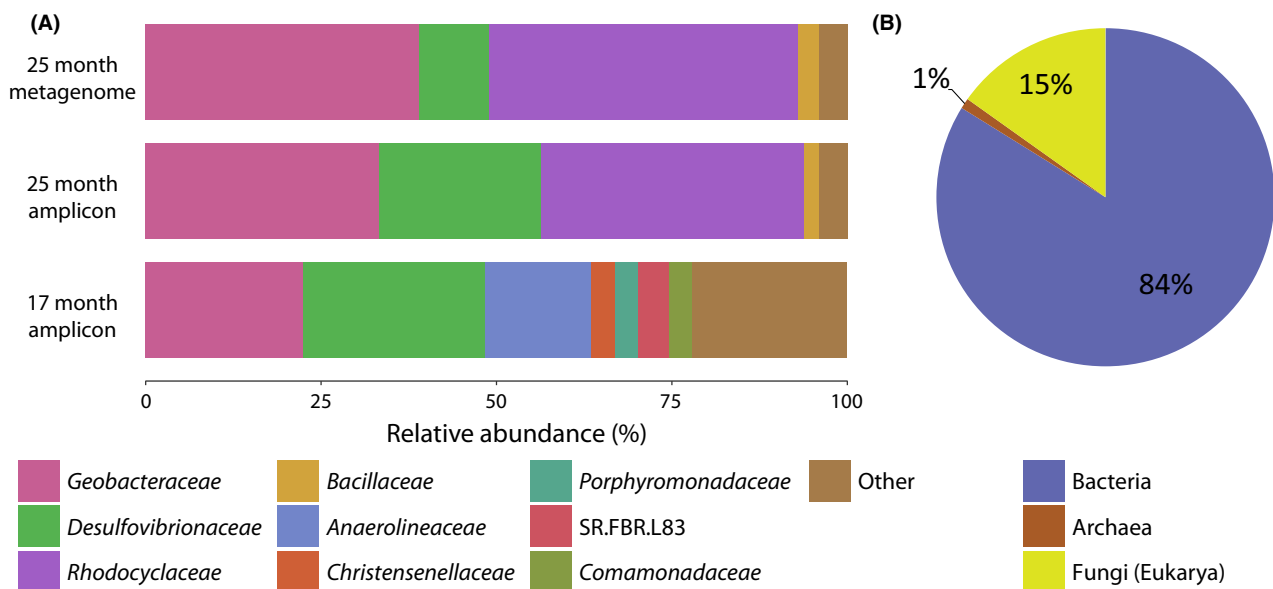
Fig. 2. Phylogenetic analysis of the microbial community at 17 and 25 months based on 16S/18S rRNA genes.
A. Bacterial taxonomy using 17 month and 25 month amplicon 16S rRNA gene data and 25 month metagenome bacterial 16S rRNA reads.
B. Relative abundance of bacteria, archaea and fungi (eukarya) based on total metagenome 16S/18S rRNA gene mapped reads at 25 months.

ecosystems (reviewed by Manohar and Raghukumar, 2013), anoxic organic carbon-rich mangrove soils (Arfi *et al.*, 2011) and anoxic freshwater sediments (Zhang *et al.*, 2015). This suggests that extremely low oxygen concentrations possibly penetrating into anoxic habitats can still support such enzyme activities. Agaricomycetes are the only class of fungi capable of substantial lignin degradation (Floudas *et al.*, 2012), which supports their potential role in the breakdown of lignin-derived structures in coal as has been showed for filamentous fungi (Hofrichter *et al.*, 1997).

Community shifts from Anaerolinaceae *to* Rhodocyclaceae *as dominant potential complex organic compound degraders*

At 17 months, the *Anaerolinaceae* were quite abundant (15% of 16S rRNA gene reads, Fig. 2) but almost completely disappeared at 25 months (< 0.2%). They were replaced by the *Rhodocyclaceae* comprising the genera *Azoarcus* and *Thauera* that were virtually absent at 17 months (< 0.4% of 16S rRNA gene reads) but became dominant community members at 25 months (23% of bacterial 16S rRNA gene reads in the amplicon survey; 44% relative abundance in the metagenome, Fig. 2). As there is no metagenome from the 17 month sample available and *Anaerolinaceae* were nearly absent at 25 months, a functional potential can only be inferred. Interestingly, their potential in coal degradation has not been suggested previously. However, *Anaerolineaceae* were dominant after long-term enrichments with

long-chain n-alkanes-dependent methanogenic conditions and occur in combination with sulfate reducers and methanogens (*Methanoculleus* and *Methanothrix*) (Liang *et al.*, 2015, 2016). In addition, enrichment from aquifer sediment with naphthalene (a polycyclic aromatic hydrocarbon) showed high relative abundance of Anaerolineaceae (5.7%–47.0%) and experimental evidence showed that polycyclic aromatic hydrocarbon (PAH) dioxygenases are present in *Rhodocyclaceae* and play a role in PAH metabolism (Singleton *et al.*, 2012; Chemerys *et al.*, 2014). The *Rhodocyclaceae* family members *Azoarcus* and *Thauera* consist of anaerobic aromatic compound degrading denitrifiers (Anders *et al.*, 1995; Liu *et al.*, 2006). As the absolute cell counts did not change considerably between the two sampling points of 17 and 25 months (Fig. 1B), it is interesting to note that the microbial community shifted towards a higher abundance of *Rhodocyclaceae*. This might indicate that the microbial community changed towards degrading complex organic compounds from coal biosolubilization. The involvement of both betaproteobacterial species in the degradation of aromatic compounds was supported by the presence of genes encoding benzylsuccinate synthase (BssABC) and 4-hydroxybenzoyl-CoA reductase (HcrAB) that previously have been shown to be involved in benzoate and toluene degradation respectively (Hermuth *et al.*, 2002; Barragán *et al.*, 2004) (Table S2). For *Azoarcus*, the functional analyses revealed the presence of benzoate-coenzyme A ligase (BzdA) which supported its capacity for benzoate degradation (Barragán *et al.*, 2004). The identification of

Thauera aromatica was possible to species level and was supported by both metagenomic 16S rRNA and functional gene analyses. Its capacity for complex organic compound degradation including relatively inert aromatic compounds may be crucial in coal breakdown processes (Hermuth *et al.*, 2002; Boll, 2011; Kuntze *et al.*, 2011). Both *Azoarcus* and *Thauera* have been observed before in sub-bituminous and bituminous methane-producing coal seams (Li *et al.*, 2008). Strikingly, we did not detect nitrate in the coal well at the start of the experiment (Table S1) so it is unclear what terminal electron acceptors could have been used by *Thauera* and *Azoarcus*.

Organic compounds could be further degraded by Geobacter metallireducens

Both the 17 and 25 month amplicon sequencing data indicated high relative abundance of *Geobacteraceae* (22% and 33% of bacterial amplicon reads, respectively, Fig. 2). The 25 month metagenomic data supported the high relative abundance (32% of 16S rRNA reads, Fig. 2) and allowed the identification of a single *Geobacter* species present, namely *Geobacter metallireducens* (100% sequence identity). *G. metallireducens* inhabits freshwater sediments and couples metal reduction of mainly iron and manganese to the complete anaerobic oxidation of a wide variety of organic electron donors including acetate, butyrate, ethanol, butanol, propanol and aromatic hydrocarbons like toluene and phenol (Lovley and Phillips, 1988; Lovley *et al.*, 2004; Aklujkar *et al.*, 2009; Kuntze *et al.*, 2011; Zhang *et al.*, 2013). In *G. metallireducens,* carbon fluxes can be balanced via the acetate kinase/phosphotransacetylase (ACK/PTA) pathway that converts acetyl-CoA to acetate with the generation of ATP, as shown for *Escherichia coli* (el-Mansi and Holms, 1989). This mechanism is present in *G. metallireducens* and is hypothesized to be used when excess organic compounds are present (Lovley and Chapelle, 1995; Aklujkar *et al.*, 2009; Speers and Reguera, 2012). Whether this pathway can provide acetate for methanogenesis in coal wells should be further investigated. Sequence analyses of metal reduction and complex organic compound degradation genes supported the potential role of *G. metallireducens* in these processes (all with 100% protein sequence identity, Table S2). Fe(III) reduction potential was analysed based on cytochromes involved in iron reduction and electron transfer mechanisms across the membrane. We found the diheme cytochrome *c* peroxidase MacA in the *G. metallireducens* draft genome (100% aa identity). In *G. sulfurreducens*, MacA is involved in the electron transfer pathway to Fe(III) (Butler *et al.*, 2004; Seidel *et al.*, 2012). We also detected the triheme cytochrome

c7 Gmet_2902 (homologous to *G. sulfurreducens* PpcA). Previous studies in both *Geobacter* species established that these cytochromes are essential for electron transfer reactions but do not directly reduce Fe(III) (Afkar and Fukumori, 1999; Lloyd *et al.*, 2003). As a candidate for Fe(III) reduction we identified a gene encoding Gmetc6, a homologue of a putative *c*-type outer membrane cytochrome (OmcF), that showed 100% protein sequence identity to the 74 aa conserved domain, indicating presence of an outer membrane electron transfer mechanism (Kim *et al.*, 2005; Mehta *et al.*, 2005). Genes encoding geopilin (PilA) and flagellar proteins for motility (FliCDS) were found and indicated the capacity for chemotaxis, direct substrate contact and importantly also electron transfer (Childers *et al.*, 2002; Tremblay *et al.*, 2012; Holmes *et al.*, 2016). These factors can provide competitive advantages in the presence of difficult to dissolve Fe(III) and Mn(IV) minerals at high pH. Together with the capacity to oxidize aromatic hydrocarbons, this supports the potential role of *Geobacter* in metal reduction in the amended coal well.

Desulfovibrio *and* Bacillus *possess the capacity to couple organic compound oxidation to iron reduction*

The amplicon data at 17 and 25 months revealed that *Desulfovibrio* accounted for 23% and 26% of the bacterial reads, respectively, whereas metagenome 16S rRNA reads indicated lower relatively lower abundance (10%) (Fig. 2). 16S rRNA and phylogenetic analysis of genes encoding sulfite reductase (DsvAB, the Dsr of *Desulfovibrio vulgaris*) revealed that *D. vulgaris* was the dominant organism. *Desulfovibrio vulgaris* belongs to a group of sulfate reducers that break down organic acids incompletely and produce acetate as end-product (Heidelberg *et al.*, 2004). However, sulfate was not detected after the first three months (< 0.02 mM) and FeS had accumulated in the sediment (data not shown). Upon sulfate depletion, *D. vulgaris* can switch to Fe(III) reduction, as well as to the reduction of Cr(VI) and U(VI) that occur as trace elements in sub-bituminous coal (Querol *et al.*, 1995; Heidelberg *et al.*, 2004). However, whether this strategy can be used by *D. vulgaris* to compete for oxidized iron and to simultaneously produce acetate as substrate for acetoclastic methanogenesis should be further investigated.

Baccillaceae were nearly absent at 17 months (0.7%) and more abundant at 25 months (2% and 5% of amplicon and metagenome 16S rRNA reads, respectively, Fig. 2) with high 16S rRNA gene sequence identity to *Bacillus subterraneus*, a species that can use Fe(III), Mn(IV), nitrate, nitrite and fumarate as electron acceptors (Kanso *et al.*, 2002; Strąpoć *et al.*, 2008). A review by Nealson and Saffarini (1994) indicated that the iron and

manganese reduction capacity of *Bacillus* species was more widespread (Nealson and Saffarini, 1994). Further work to assess coupling of metal reduction to organic compounds degradation and growth in *Bacillus* species is required.

Acetoclastic Methanothrix *(formerly:* Methanosaeta*) species are responsible for methanogenesis*

Both at 17 and 25 months, *Methanosaetaceae* were the dominant archaeal family ($\geq$ 99%). Based on the relative abundance data from the metagenome, they constitute 1% of the total microbial community (Fig. 2). Methane concentration measurements indicated highest methanogenic activity between 12 and 19 months after amendment. All members of the family *Methanosaetaceae* are strictly acetoclastic methanogens, indicating that hydrogenotrophic methanogenesis does not play a significant role in the amended coal well. BLASTX analysis of raw and *de novo* assembled reads that mapped to the methyl-coenzyme M reductase protein subunit A (McrA) sequence database indicated high identity to both *Methanothrix concilii* GP-6 (1 contig with 94% protein sequence identity) and uncultured methanogens predominantly found in lake sediments (Denonfoux *et al.*, 2013; Youngblut *et al.*, 2014; Lin *et al.*, 2015; Pump *et al.*, 2015). More specifically, the one assembled contig was very similar to the McrA sequence of an uncultured *Methanothrix* sp. from 90 m deep Lake Pavin sediment (95% protein sequence identity) (Denonfoux *et al.*, 2013), in line with high similarity of the 16S rRNA gene sequence to uncultured *Methanothrix* species from Lake Pavin sediment and water samples (Lehours *et al.*, 2005, 2007; Borrel *et al.*, 2012). It is interesting to note that *Methanothrix* co-occurred with *Geobacter*. A previous study by Jones *et al.* (2010) on bioaugmentation of sub-bituminous coal showed degradation of single-ring aromatics and long-chain alkanes and subsequent methane production by a *Geobacter* spp. and *Methanothrix concilii* dominated culture. Experiments on microbial aggregates and co-cultures showed that *Methanothrix-Geobacter* clusters are electrically conductive which suggested that direct interspecies electron transfer (DIET) plays an important role (Summers *et al.*, 2010; Morita *et al.*, 2011; Rotaru *et al.*, 2014; Holmes *et al.*, 2017). These processes could also be relevant in the acetate and nutrient amended coal well.

A community with low complexity has the potential for methanogenesis from coal but does not lead to successful continued methane production

Using geochemical, 16S rRNA gene amplicon and metagenome sequencing, we established that the microbial community in a nutrient and acetate amended Australian coal well had the potential to break down coal to methane (Fig. 3). The methane production data (Fig. 1A) indicated that methane was generated by acetate conversion rather than by coal degradation. It is puzzling that the microbial community primarily consisted of complex organic compound degraders, which would not have an apparent role in the conversion of acetate to methane. The second key observation is that nutrient amendment lead to an increase in microbial cell counts by four orders of magnitude, which did not result in direct methane production from coal. Degradation of dead microbial biomass could also play a role, but is unlikely to serve as a major organic compound source for methanogenesis. Both observations make it questionable whether nutrient and acetate amendment will help to sustainably generate coalbed methane from non-producing coal wells, even if such amendments stimulate the microbial growth in general and help to bioaugment relevant microorganisms such as acetoclastic methanogens or complex organic compound degraders. In general, biological methane production from coal is a promising
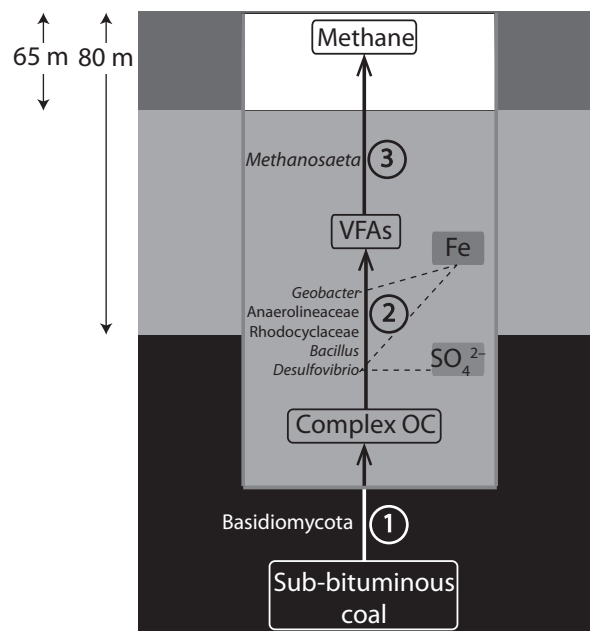
Fig. 3. Proposed scheme illustrating the potential of the microbial community to convert sub-bituminous coal to methane. The groundwater level is 65 m below ground level and the coal bearing layers are located 80 m below ground level. Fungi related to the Basidiomycota possess the capacity for coal biosolubilization. The bacterial community has the capacity for complex organic compounds degradation to volatile fatty acids (VFAs). Iron was detected as potential electron acceptor for *Desulfovibrio* and *Geobacter*, and sulfate could play a role only in the first three months. Acetoclastic *Methanothrix* spp. were the dominant methanogens in the coal well. Although the potential for bioconversion of the coal matrix was found, coalbed methanogenesis could not be observed.

alternative to direct coal burning as it reduces inorganic and residual pollution in the form of heavy metals and sulfur compounds. From our microbial food web analysis coupled to the functional output of the nutrient and acetate amended coal well, it is apparent that it is not straightforward to link metabolic potentials to a system output. More *in situ* data on microbial activity, functioning and nutrient cycling are required to understand and overcome bottlenecks in the biomethanation process from coal.

Experimental procedures

Sample site

The two compared coal wells are located at the Lithgow State Coal Mine in the Western Coal Fields of New South Wales, Australia (Fig. S1). 30.6% of the raw subbituminous coal in this study consisted of volatile hydrocarbons; over 50% consisted of fixed carbon (Mark Wainwright Analytical Centre, UNSW, Sydney, Australia). NH_4Cl and Na_2PO_4 for the amendments were purchased from Ajax Finechem, Scoresby VIC, Australia and Sigma-Aldrich, Castle Hill NSW, Australia respectively. For the nutrient and acetate additions, a 100 m × 0.5 mm (length × diameter) silicon tube was lowered to the base of the well (80 m below ground level) and concentrated nutrient solutions were gravity fed (siphoned) into the subsurface in syringe-fed portions of 100 ml. During the feed, the tube was repeatedly manually raised and lowered from the bottom to the top of the water column (approximately 20 m) ensuring even distribution over the height of the water column. Lateral mixing was achieved by passive diffusion and disturbances imposed in sampling operations. By gravity flow the feeding rate was about 0.5 L min^{-1}. Water sampling was performed using a well bladder pump (PVC 3/8 in discharge, ThermoFisher Scientific, Scoresby VIC, Australia) deployed with a stainless steel drop tube and a low refill ratio of 40:20 to avoid the removal of dissolved gases in the formation water. The drop tube was lowered to a depth of 75–78 m. The pump and connecting tubes were drained between the wells to avoid carriage of water between well sampling. Formation water for chemical and microbiological analyses was sampled monthly over the 25 month operation period. Samples were immediately processed as described below.

Ionic composition of the well water

Analyses of cation and anion concentrations were carried out at the Mark Wainwright Centre (University of New South Wales, Sydney NSW, Australia). Briefly, cation (B, Ca, Fe, K, Mg, Mn, Na, P, S, Si) concentrations were analysed using inductive coupled plasma optical emission spectroscopy (ICP-OES, Optima7300DV, Perkin-Elmer, Waltham MA, USA) with a Segmented-array Charged-coupled Device (SCD, Perkin-Elmer). The setting of the instrument was as follows: forward power 1200–1400 W, reflected power 20.0 W, nebulizer gas flow of 0.7 L min^{-1}, plasma gas flow of 10.0–15.0 L min^{-1}, aux gas flow of 0.3 L min^{-1}. Anion (F, Cl, Br, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}) concentrations were analysed using ion chromatography (IC, Dionex ICS1000, Dionex Corporation, Sunnyvale CA, USA) with conductivity detection using an Ion Pac AS14-4 mm column (Dionex Corporation) and a mobile phase of 0.8 M Na_2CO_3/0.1 M $NaHCO_3$. The instrument detection limits were 0.02 mg L^{-1} for B, Si and Mn, 0.2 mg L^{-1} for Fe and P, 0.5 mg L^{-1} for Ca, K, Mg, Na and S, 2 mg L^{-1} for F, NO_2^-, NO_3^- and Br, and 5 mg L^{-1} for Cl and PO_4^{3-}.

Total methane and acetate analyses

Total methane (headspace and dissolved) and acetate concentrations were monitored monthly over a period of 25 months. Gas samples were taken from the well-head apertures and were transferred directly into 10 ml gastight serum vials using a gastight glass syringe. Dissolved methane in 100 ml sampled coal formation water was analysed according to Kampbell and Vandegrift (1998) with modifications. Briefly, formation water was sampled anoxically into 120 ml nitrogen degassed serum vials containing 1 ml of formic acid (Sigma-Aldrich, Castle Hill NSW, Australia). Subsequently, samples were equilibrated to room temperature (20°C), and a 10 ml headspace was created in the vials by replacing 10 ml of liquid with 10 ml of nitrogen using a gastight glass syringe. Vials were inverted and equilibrated for 24 h. Methane from the headspace was analysed using a Shimadzu GC-2010 gas chromatograph with flame ionization detection (GC-FID) fitted with a GASPRO PLOT column (60 m × 0.32 mm; Agilent Technologies, Mulgrave VIC, Australia). The carrier gas was helium (3 ml min^{-1}), and inlet temperature was 250°C. Oven temperature program: isothermal 100°C (1 min) and then 25°C min^{-1} to 250°C and held for 1 min. Gas samples of 100 μl were withdrawn directly from the sampling flasks using a pressure lockable gastight glass syringe (SGE Analytical Science, Ringwood VIC, Australia) and injected into the GC. Compounds were quantified by comparison of the peak area of the unknown with an eight-point calibration curve with a lower detection limit of 0.2 μM. Calibration standards were made up in 60 ml serum vials.

For the analyses of acetate concentrations, water was filtered through a 0.2 μm syringe filter (Merck Millipore, Bayswater VIC, Australia) and subsequently 900 μl

filtered water was acidified with 100 μl formic acid (10% v/v; Sigma-Aldrich, Castle Hill NSW, Australia) to a pH < 2. Acetate (1 μl) was analysed by GC-FID (Shimadzu, Rydalmere NSW, Australia) using a DB-FFAP column (30 m × 0.32 mm; Agilent Technologies, Mulgrave, Australia) with helium (1 mL min^{-1}) as carrier gas. Injection port was set at 250°C with split mode (1:30). Oven temperature was set to 60°C for 1 min and then 15°C min^{-1} to 250°C.

Cell counts

Coal formation water samples were immediately fixed by the addition of glutaric dialdehyde (0.2 μm filtered, 2% final concentration) and stored at 4°C in the dark. Prior to analysis, an aliquot was diluted 1000-fold in particle-free phosphate-buffered saline (0.9 g of NaCl, sodium phosphate buffer 15 m$_M$, pH 7.4, 0.2 μm filtered), thoroughly shaken and transferred to a microscopic slide that was treated with a mounting medium (9.6% Mowiol 4-88, Sigma-Aldrich, Castle Hill NSW, Australia and 24% glycerol) prior to applications. Cells were stained using SybrGreen I (Sigma-Aldrich, Castle Hill NSW, Australia), and counting was performed using a BX51 epifluorescence microscopy (Olympus, Notting Hill VIC, Australia) as described by Lunau *et al.* (2005).

DNA extraction

After 17 and 25 months, DNA was extracted from 2 g of well sediment using the phenol/chloroform extraction method as described by Lueders and co-workers (Lueders *et al.*, 2004). Subsequently, DNA was precipitated using polyethylene glycol 6000 (Sigma-Aldrich, St Louis MI, United States), and the DNA pellet was washed once with 70% ethanol and dissolved in 50 μl elution buffer (Qiagen, Venlo, the Netherlands). Three parallel extractions were carried out, and extracts were pooled for each incubation treatment. DNA concentration and purity were determined by standard agarose gel electrophoresis and fluorometrically using RiboGreen assays (Qubit Assay Kit, Invitrogen, Waltham MA, United States) according to the manufacturer's instructions.

DNA sequencing and data processing

Both the 17 and 25 month DNA samples were used for 16S rRNA gene amplicon sequencing on the IonTorrent PGM™. Amplification of the V3-V4 region of the bacterial 16S rRNA gene was performed using universal primers Bac F341 (5′-CCTACGGGNGGCWGCAG-3′) and Bac785R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth *et al.*, 2013) for 25 cycles. Archaeal 16S rRNA genes were amplified with the universal archaeal primers Archf349 (5′- GYGCASCAGKCGMGAAW-3′) and Archr 789 (5′-GGACTACVSGGGTATCTAAT-3′) (Klindworth *et al.*, 2013) for 30 cycles. Both PCR amplifications were performed by 10 min 98°C initialization, 25/30 cycles of 1 min denaturation at 95°C, 1 min of annealing at 60°C, 2 min of elongation at 72°C and a 10 min final elongation step at 72°C. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Venlo, the Netherlands) in two elution steps. 20 μl 55°C Milli-Q was added to the spin column and incubated for 2 min prior to centrifugation. Next, the eluate was put onto the spin column, incubated at 55°C for 2 min and centrifuged again as described in the manual. A 10 cycle nested PCR with IonTorrent adapters was performed on the purified PCR products using the same PCR protocol. After PCR purification with the QIAquick PCR Purification Kit as described above, PCR products were used for library preparation and sequencing steps according to the manufacturer's instructions (Life Technologies, Carlsbad CA, United States). Amplicon sequences were quality checked for chimeras and clustered into OTUs with a 97% identity cut-off value using the 454 SOP (http://www.mothur.org/) (Schloss *et al.*, 2009) with IonTorrent modified protocols. Chimeras were checked with the Uchime algorithm (Edgar *et al.*, 2011). Taxonomy was assigned against the SILVA nr v123 database using the MOTHUR taxonomy assigner (Schloss *et al.*, 2009). Data visualization was performed using the 'VEGAN' package in R (Oksanen *et al.*, 2017).

Metagenome sequencing of the 25-month DNA sample was performed on the IonTorrent PGM™. All library preparation and sequencing steps were performed according to the manufacturer's instructions (Ion PGM™ Template OT2 400 Kit, Life Technologies, Carlsbad CA, United States). 100 ng genomic DNA was sheared in four-one minute shear one minute cool down cycles using a Bioruptor® Sonicator (Diagenode, Denville NJ, United States). The library was prepared using the Ion Plus Fragment Library Kit protocol. Size distribution and concentration of the library was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara CA, United States). Sequencing was performed using the Ion 318 Chip Kit v2.

Raw reads were quality checked using the CLC Genomics Workbench 8.0 (CLCbio, Aarhus, Denmark). Ambiguous sequences were trimmed off using an ambiguous limit of 2 and a trim limit of 0.5. On both the 3′ and 5′ sides, the three terminal nucleotides were discarded. To reduce noise, reads were subsequently filtered, targeting reads with a length between 30 and 400 nt (5 153 485 reads) for *de novo* assembly and 100–400 nt (4 884 119 reads) for 16S rRNA and functional gene analysis. For *de novo* assembly, minimum contig size was set to 1000, word size to 35 and bubble

size to 5000. Values for mismatch, insertion and deletion cost were set to 2, 3 and 3, respectively, resulting in a total of 12 527 contigs with an average length of 2624 base pairs. 85% of the reads (1.1 Gbp) were assigned to a specific contig based on a length and similarity fraction of 50% and 80% respectively.

Analysis of environmental genomes

The assembled contig data set was analysed based on average read coverage and GC content per contig using R (https://www.r-project.org/) (R Core Team, 2014) and RSTUDIO v3 (https://www.rstudio.com/) (RStudio Team, 2014) with packages 'GGPLOT2' (Wickham, 2009) and 'RCOLORBREWER 1.1-2' (Neuwirth, 2011) (https://cran.r-project.org/web/packages/). The *Geobacter* environmental draft genome bin was extracted based on a GC content between 0.525 and 0.65 and a sequencing depth between 100 and 325. The resulting bin contained 139 contigs of which 118 were most identical to *Geobacter metallireducens* GS-15 (97% of bases, 3.4 Mbp, ≥ 99.9% nt identity). CheckM (Parks *et al.*, 2015) confirmed presence of a single, 91.0% complete non-heterogeneous strain. Identity of the draft genome contigs was determined by performing nucleotide BLAST searches against the nt/nr collection database. The methanogen environmental draft genome bin was extracted based on a GC content between 0.475 and 0.575 and a sequencing depth between 3 and 10. The resulting bin contained 459 contigs of which 353 contigs were most identical to *Methanothrix concilii* GP6 (86% of bases, 2.3 Mbp, ≥ 79.9% nt identity). CheckM showed strain heterogeneity of 11.1% and completeness of 76.8%, indicating the bin likely contains a core genome of *Methanothrix* species. Nucleotide BLAST on nine contigs with sequencing depth > 500 indicated best hits to *Geobacter metallireducens* GS-15 (six sequences, ≥ 99% identity) and *Azoarcus* sp. (three sequences, ≥ 94% identity).

Phylogenetic 16S and 18S rRNA gene analysis

For the general species composition analysis, quality trimmed reads were mapped against the Silva SSU non-redundant database version 128 (https://www.arb-silva.de/) containing 645 151 reference sequences. Length and similarity fraction were set to 50% and 70%, respectively, and values for mismatch, insertion and deletion cost to 2, 3 and 3, resulting in a total of 28 774 mapped reads (0.59% of total). Sequences were submitted to the SILVANGS data analysis server 1.3.5 (Quast *et al.*, 2012) and processed using default parameters. The species distribution pattern remains similar when additionally correcting for 16S rRNA gene copy numbers that are high in the Proteobacteria and Firmicutes phyla (Klappenbach *et al.*, 2001; Acinas *et al.*, 2004). Reads mapping to the SSU database were extracted per genus. *De novo* assembly was performed using automatic word and bubble size and stringent similarity and length fraction parameters of 0.95 to obtain near-complete 16S sequences. Comparable approaches were taken previously (Bartram *et al.*, 2011; Miller *et al.*, 2011; Speth *et al.*, 2016). Additionally, quality trimmed reads were mapped against the fungal internal transcribed spacer (ITS) RefSeq Targeted Loci database containing 5362 sequences (PRJNA177353) as described above (Alvarez *et al.*, 2017).

Functional analyses

Draft genome and metagenome annotations were performed using PROKKA version 1.10 with standard parameters (Seemann, 2014). The *Geobacter* environmental draft genome was first annotated against a *Geobacter* reference data set containing nine annotated genomes. Contig sequences were uploaded to the KEGG Automated Annotation Server (KAAS) (Moriya *et al.*, 2007) and analysed using 40 most related reference organisms. Specific protein sequence data sets for metal reduction, complex organic compounds degradation and genus specific genes were manually selected and downloaded from the UNIPROT database (http://www.uniprot.org/) (The UniProt Consortium, 2014). For the analysis of the fungal community, fungal peroxidase databases were made for lignin, manganese and versatile peroxidases by downloading nucleotide data from the NCBI database, filtering for fungal sequences ≤ 100 000 bp. Reference databases were imported in CLC and mapped against the genomic data sets using the CLC BLASTX tool using the standard parameters: expect: 10.0, match: 2, mismatch: -3, gap costs existence: 5, gap cost extension: 2. Artemis 16.0.0 was used for visualization of the annotation results (Rutherford *et al.*, 2000). Methanogen and methanotroph functional gene analyses were performed using McrA, MmoX and PmoA/AmoA protein sequence databases and a non-redundant reference database (Claudia Lüke, Radboud University, personal communication). Analysis of trimmed reads was performed using the BLASTX (BLAST 2.2.25+) algorithm with *e*-value 0.0001 extracting first hits as target sequence. Functional gene and non-redundant BLAST results with a bit-score > 125 were merged. All reads had a BLAST score ratio of > 0.8 (Lüke *et al.*, 2016). Only McrA analysis resulted in significant hits.

Nucleotide sequence accession numbers

All sequencing data were submitted to the GenBank databases under the BioProject PRJNA321375. The assembled

IonTorrent data set from this study was submitted as Sequence Read Archive study under accession number SRP074879 (run SRR5328925). The amplicon data were submitted as SAMN07344736 (17 month archaeal), SAMN07344812 (17 month bacterial), SAMN07344862 (25 month archaeal) and SAMN07344863 (25 month bacterial).

Conflict of interest

None declared.

References

Acinas, S. G., Marcelino, L. A., Klepac-Ceraj, V., and Polz, M. F. (2004) Divergence and redundancy of 16S rRNA sequences in genomes with multiple *rrn* operons. *J Bacteriol* **186:** 2629–2635.

Afkar, E., and Fukumori, Y. (1999) Purification and characterization of triheme cytochrome c7 from the metal-reducing bacterium, *Geobacter metallireducens*. *FEMS Microbiol Lett* **175:** 205–210.

Aklujkar, M., Krushkal, J., DiBartolo, G., Lapidus, A., Land, M. L., and Lovley, D. R. (2009) The genome sequence of *Geobacter metallireducens*: features of metabolism, physiology and regulation common and dissimilar to *Geobacter sulfurreducens*. *BMC Microbiol* **9:** 1–22.

Al-Jubori, A., Johnston, S., Boyer, C., Lambert, S. W., Bustos, O. A., Pashin, J. C., and Wray, A. (2009) Coalbed methane: clean energy for the world. *Oilfield Rev* **21:** 4–13.

Alvarez, L. V., Groenewald, J. Z., and Crous, P. W. (2017) Revising the Schizoparmaceae: *Coniella* and its synonyms *Pilidiella* and *Shizoparme*. *Stud Mycol* **85:** 1–34.

Anders, H. J., Kaetzke, A., Kämpfer, P., Ludwig, W., and Fuchs, G. (1995) Taxonomic position of aromatic-degrading denitrifying *Pseudomonas strains* K 172 and KB 740 and their description as new members of the genera *Thauera*, as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov. *Int J Syst Bacteriol* **45:** 327–333.

Arfi, Y., Marchand, C., Wartel, M., and Record, E. (2011) Fungal diversity in anoxic-sulfidic sediments in a mangrove soil. *Fungal Ecol* **5:** 282–285.

Ball, A., Ahmad, S., McCluskey, C., Pham, P., Ahn, I., Dawson, L., *et al.* (2016) Austrialian Energy Update 2016.

Barragán, M. J. L., Carmona, M., María, T., Thiele, B., Boll, M., Fuchs, G., *et al.* (2004) The bzd gene cluster coding for anaerobic benzoate catabolism in *Azoarcus* sp. strain CIB. *J Bacteriol* **186:** 5762–5774.

Bartram, A. K., Lynch, M. D. J., Stearns, J. C., Moreno-Hagelsieb, G., and Neufeld, J. D. (2011) Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. *Appl Environ Microbiol* **77:** 3846–3852.

Boll, M. (2011) 4-Hydroxybenzoyl-coenzyme A reductase. In *Encyclopedia of Inorganic and Bioinorganic Chemistry*. Hoboken: John Wiley & Sons Ltd.

Borrel, G., Colombet, J., Robin, A., Lehours, A. C., Prangishvili, D., and Sime-Ngando, T. (2012) Unexpected and novel putative viruses in the sediments of a deep-dark permanently anoxic freshwater habitat. *ISME J* **6:** 2119–2127.

Butler, J. E., Kaufmann, F., Coppi, M. V., and Nu, C. (2004) MacA, a diheme c-type cytochrome involved in Fe(III) reduction by *Geobacter sulfurreducens*. *J Bacteriol* **186:** 4042–4045.

Chemerys, A., Pelletier, E., Cruaud, C., Martin, F., Violet, F., and Jouanneau, Y. (2014) Characterization of novel polycyclic aromatic hydrocarbon dioxygenases from the bacterial metagenomic DNA of a contaminated soil. *Appl Environ Microbiol* **80:** 6591–6600.

Childers, S. E., Ciufo, S., and Lovley, D. R. (2002) *Geobacter metallireducens* accesses insoluble Fe(III) oxide by chemotaxis. *Nature* **416:** 767–769.

Day, S., Dell'Amico, M., Fry, R., and Tousi, H. (2014) *Field Measurements of Fugitive Emissions from Equipment and Well Casings in Australian Coal Seam Gas Production Facilities*. Canberra: CSIRO.

Denonfoux, J., Parisot, N., Dugat-Bony, E., Biderre-Petit, C., Boucher, D., Morgavi, D. P., *et al.* (2013) Gene capture coupled to high-throughput sequencing as a strategy for targeted metagenome exploration. *DNA Res* **20:** 185–196.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27:** 2194–2200.

Faiz, M., and Hendry, P. (2006) Significance of microbial activity in Australian coal bed methane reservoirs – a review. *Bull Can Pet Geol* **54:** 261–272.

Fakoussa, R., and Hofrichter, M. (1999) Biotechnology and microbiology of coal degradation. *Appl Microbiol Biotechnol* **52:** 25–40.

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R., Herissat, B., *et al.* (2012) The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. *Science* **336:** 1715–1720.

Heidelberg, J. F., Seshadri, R., Haveman, S. A., Hemme, C. L., Paulsen, I. T., Kolonay, J. F., *et al.* (2004) The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Nat Biotechnol* **22:** 554–559.

Hermuth, K., Leuthner, B., and Heider, J. (2002) Operon structure and expression of the genes for benzylsuccinate synthase in *Thauera aromatica* strain K172. *Arch Microbiol* **177:** 132–138.

Hofrichter, M., Bublitz, F., and Fritsche, W. (1997) Fungal attack on coal II. Solubilization of low-rank coal by filamentous fungi. *Fuel Process Technol* **52:** 55–64.

Holmes, D. E., Dang, Y., Walker, D. J. F., and Lovley, D. R. (2016) The electrically conductive pili of *Geobacter* species are a recently evolved feature for extracellular electron transfer. *Microbial Genomics* **2:** 1–20.

Holmes, D. E., Shrestha, P. M., Walker, D. J. F., Dang, Y., Nevin, K. P., Woodard, T. L., and Lovley, D. R. (2017) Metatranscriptomic evidence for direct interspecies electron transfer between *Geobacter* and *Methanothrix* species in methanogenic rice paddy soils. *Appl Environ Microbiol* **2:** AEM.00223-17.

Iram, A., Akhtar, K., and Ghauri, M. A. (2017) Coal methanogenesis: a review of the need of complex microbial consortia and culture conditions for the effective bioconversion of coal into methane. *Ann Microbiol* **67:** 275–286.

Jones, E. J. P., Voytek, M. A., Corum, M. D., and Orem, W. H. (2010) Stimulation of methane generation from nonproductive coal by addition of nutrients or a microbial consortium. *Appl Environ Microbiol* **76:** 7013–7022.

Kampbell, D. H., and Vandegrift, S. A. (1998) Analysis of dissolved methane, ethane, and ethylene in ground water by a standard gas chromatographic technique. *J Chromatogr Sci* **36:** 253–256.

Kanso, S., Greene, A. C., and Patel, B. K. C. (2002) *Bacillus subterraneus* sp. nov., an iron- and manganese-reducing bacterium from a deep subsurface Australian thermal aquifer. *Int J Syst Evol Microbiol* **52:** 869–874.

Kim, B., Leang, C., Ding, Y., Glaven, R., Coppi, M., and Lovley, D. (2005) OmcF, a putative c-type monoheme outer membrane cytochrome required for the expression of other outer membrane cytochromes in *Geobacter sulfurreducens*. *J Bacteriol* **187:** 4505–4513.

Kinnon, E. C. P., Golding, S. D., Boreham, C. J., Baublys, K. A., and Esterle, J. S. (2010) Stable isotope and water quality analysis of coal bed methane production waters and gases from the Bowen Basin, Australia. *Int J Coal Geol* **82:** 219–231.

Klappenbach, J. A., Saxman, P. R., Cole, J. R., and Schmidt, T. M. (2001) rrndb: the ribosomal RNA operon copy number database. *Nucleic Acids Res* **29:** 181–184.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., and Glöckner, F. O. (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* **41:** 1–11.

Kuntze, K., Vogt, C., Richnow, H., and Boll, M. (2011) Combined application of PCR-based functional assays for the detection of aromatic-compound-degrading anaerobes. *Appl Environ Microbiol* **77:** 5056–5061.

Laborda, F., Fernández, M., Luna, N., and Monistrol, I. F. (1997) Study of the mechanisms by which microorganisms solubilize and/or liquefy Spanish coals. *Fuel Process Technol* **52:** 95–107.

Lehours, A., Bardot, C., Thenot, A., Fonty, G., and Debroas, D. (2005) Anaerobic microbial communities in Lake Pavin, a unique meromictic lake in France. *Appl Environ Microbiol* **71:** 7389–7400.

Lehours, A., Evans, P., Bardot, C., Joblin, K., and Gérard, F. (2007) Phylogenetic diversity of archaea and bacteria in the anoxic zone of a meromictic lake (Lake Pavin, France). *Appl Environ Microbiol* **73:** 2016–2019.

Li, D., Hendry, P., and Faiz, M. (2008) A survey of the microbial populations in some Australian coalbed methane reservoirs. *Int J Coal Geol* **76:** 14–24.

Liang, B., Wang, L.-Y., Mbadinga, S. M., Liu, J.-F., Yang, S.-Z., Gu, J.-D., and Mu, B.-Z. (2015) *Anaerolineaceae* and *Methanosaeta* turned to be the dominant microorganisms in alkanes-dependent methanogenic culture after long-term of incubation. *AMB Express* **5:** 37.

Liang, B., Wang, L. Y., Zhou, Z., Mbadinga, S. M., Zhou, L., Liu, J. F., et al. (2016) High frequency of *Thermodesulfovibrio* spp. and *Anaerolineaceae* in association with methanoculleus spp. in a long-term incubation of n-alkanes-degrading methanogenic enrichment culture. *Front Microbiol* **7:** 1–13.

Lin, Y., Liu, D., Ding, W., Kang, H., Freeman, C., Yuan, J., and Xiang, J. (2015) Substrate sources regulate spatial variation of metabolically active methanogens from two contrasting freshwater wetlands. *Appl Microbiol Biotechnol* **99:** 10779–10791.

Liu, B., Zhang, F., Feng, X., Liu, Y., Yan, X., Zhang, X., et al. (2006) Thauera and *Azoarcus* as functionally important genera in a denitrifying quinoline-removal bioreactor as revealed by microbial community structure comparison. *FEMS Microbiol Ecol* **55:** 274–286.

Lloyd, J. R., Leang, C., Hodges Myerson, A. L., Coppi, M. V., Cuifo, S., Methe, B., et al. (2003) Biochemical and genetic characterization of PpcA, a periplasmic c-type cytochrome in *Geobacter sulfurreducens*. *Biochem J* **369:** 153–161.

Lovley, D., and Chapelle, F. (1995) Deep subsurface microbial processes. *Rev Geophys* **33:** 365–381.

Lovley, D., and Phillips, E. (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl Environ Microbiol* **54:** 1472–1480.

Lovley, D., Holmes, D., and Nevin, K. (2004) Dissimilatory Fe (III) and Mn(IV) reduction. *Adv Microb Physiol* **49:** 219–286.

Lueders, T., Manefield, M., and Friedrich, M. W. (2004) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6:** 73–78.

Lüke, C., Speth, D. R., Kox, M. A. R., Villanueva, L., and Jetten, M. S. M. (2016) Metagenomic analysis of nitrogen and methane cycling in the Arabian Sea oxygen minimum zone. *PeerJ* **4:** e1924.

Lunau, M., Lemke, A., Walther, K., Martens-Habbena, W., and Simon, M. (2005) An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Environ Microbiol* **7:** 961–968.

Manohar, C. S., and Raghukumar, C. (2013) Fungal diversity from various marine habitats deduced through culture-independent studies. *FEMS Microbiol Lett* **341:** 69–78.

el-Mansi, E. M., and Holms, W. H. (1989) Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. *Microbiology* **135:** 2875–2883.

Mayumi, D., Mochimaru, H., Yoshioka, H., Sakata, S., Maeda, H., Miyagawa, Y., et al. (2011) Evidence for syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis in the high-temperature petroleum reservoir of Yabase oil field (Japan). *Environ Microbiol* **13:** 1995–2006.

Mayumi, D., Mochimaru, H., Tamaki, H., Yamamoto, K., Yoshioka, H., Suzuki, Y., et al. (2016) Methane production from coal by a single methanogen. *Science* **354:** 222–225.

Mehta, T., Coppi, M., Childers, S., and Lovley, D. (2005) Outer membrane c -type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. *Appl Environ Microbiol* **71:** 8634–8641.

Midgley, D. J., Hendry, P., Pinetown, K. L., Fuentes, D., Gong, S., Mitchell, D. L., and Faiz, M. (2010) Characterisation of a microbial community associated with a deep, coal seam methane reservoir in the Gippsland Basin, Australia. *Int J Coal Geol* **82:** 232–239.

Miller, C. S., Baker, B. J., Thomas, B. C., Singer, S. W., and Banfield, J. F. (2011) EMIRGE: reconstruction of full-length ribosomal genes from microbial community short read sequencing data. *Genome Biol* **12:** 1–14.

Moore, T. J. (2012) Coalbed methane: a review. *Int J Coal Geol* **101:** 36–81.

Morita, M., Malvankar, N. S., Franks, A. E., Summers, Z. M., Giloteaux, L., Rotaru, A. E., *et al.* (2011) Potential for direct interspecies electron transfer in methanogenic wastewater digester aggregates. *mBio* **2:** 5–7.

Moriya, Y., Itoh, M., Okuda, S., Yoshizawa, A. C., and Kanehisa, M. (2007) KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic Acids Res* **35:** 182–185.

Myhre, G., Shindell, D., Bréon, F.-M., Collins, W., Fuglestvedt, J., Huang, J., *et al.* (2013) Anthropogenic and natural radiative forcing. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Stocker, T. F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S. K., Boschung, J., Nauels, A., Xia, Y., Bex, V., and Midgley, P. M. (eds). Cambridge, UK and New York, NY: Cambridge University Press, pp. 713–714.

Nealson, K. H., and Saffarini, D. (1994) Iron and manganese in anaerobic respiration: environmental significance, physiology, and regulation. *Annu Rev Microbiol* **48:** 311–343.

Neuwirth, E. (2011) RColorBrewer: ColorBrewer Palettes. [WWW document]. https://CRAN.R-project.org/package= RColorBrewer.

Ohm, R. A., Riley, R., Salamov, A., Min, B., Choi, I., and Grigoriev, I. V. (2014) Genomics of wood-degrading fungi. *Fungal Genet Biol* **72:** 82–90.

Oksanen, A. J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., *et al.* (2017) Package "vegan", community ecology package. [WWW document]. https:// CRAN.R-project.org/package=vegan.

Papendick, S. L., Downs, K. R., Vo, K. D., Hamilton, S. K., Dawson, G. K. W., Golding, S. D., and Gilcrease, P. C. (2011) Biogenic methane potential for Surat Basin, Queensland coal seams. *Int J Coal Geol* **88:** 123–134.

Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., and Tyson, G. W. (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* **25:** 1043–1055.

Penner, T. J., Foght, J. M., and Budwill, K. (2010) Microbial diversity of western Canadian subsurface coal beds and methanogenic coal enrichment cultures. *Int J Coal Geol* **82:** 81–93.

Pump, J., Pratscher, J., and Conrad, R. (2015) Colonization of rice roots with methanogenic archaea controls photosynthesis-derived methane emission. *Environ Microbiol* **17:** 2254–2260.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., *et al.* (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41:** 1–7.

Querol, X., Fernhndez-Turiel, J. L., and Lbpez-Soler, A. (1995) Trace elements in coal and their behaviour during combustion in a large power station. *Fuel* **74:** 331–343.

R Core Team (2014) *R: A Language and Environment for Statistical Computing*. Vienna: The R Foundation for Statistical Computing.

Robbins, S. J., Evans, P. N., Esterle, J. S., Golding, S. D., and Tyson, G. W. (2016) The effect of coal rank on biogenic methane potential and microbial composition. *Int J Coal Geol* **154–155:** 205–212.

Rotaru, A.-E., Shrestha, P. M., Liu, F., Shrestha, M., Shrestha, D., Embree, M., *et al.* (2014) A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the reduction of carbon dioxide to methane. *Energy Environ Sci* **7:** 408–415.

RStudio Team (2014) *RStudio: Integrated Development for R*. Boston: RStudio.

Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M.-A., and Barrell, B. (2000) Artemis: sequence visualization and annotation. *Bioinformatics* **16:** 944–945.

Saunois, M., Bousquet, P., Poulter, B., Peregon, A., Ciais, P., Canadell, J. G., *et al.* (2016) The global methane budget 2000–2012. *J Earth Syst Sci* **8:** 697–751.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., *et al.* (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75:** 7537–7541.

Seemann, T. (2014) Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30:** 2068–2069.

Seidel, J., Hoffmann, M., Ellis, K. E., Seidel, A., Spatzal, T., Gerhardt, S., *et al.* (2012) MacA is a second cytochrome c peroxidase of *Geobacter sulfurreducens*. *Biochemistry* **51:** 2747–2756.

Singleton, D. R., Hu, J., and Aitken, M. D. (2012) Heterologous expression of Polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenase genes from a novel pyrene-degrading betaproteobacterium. *Appl Environ Microbiol* **78:** 3552–3559.

Speers, A. M., and Reguera, G. (2012) Electron donors supporting growth and electroactivity of *Geobacter sulfurreducens* anode biofilms. *Appl Environ Microbiol* **78:** 437–444.

Speth, D. R., in 't Zandt, M. H., Guerrero-Cruz, S., Dutilh, B. E., and Jetten, M. S. M. (2016) Genome-based microbial ecology of anammox granules in a full-scale wastewater treatment system. *Nat Commun* **7:** 1–10.

Strąpoć, D., Picardal, F., Turich, C., Schaperdoth, I., Macalady, J., Lipp, J., *et al.* (2008) Methane-producing microbial community in a coal bed of the Illinois basin. *Appl Environ Microbiol* **74:** 2424–2432.

Summers, Z. M., Fogarty, H. E., Leang, C., Franks, A. E., Malvankar, N. S., and Lovley, D. R. (2010) Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. *Science* **330:** 1413–1415.

The UniProt Consortium (2014) UniProt: a hub for protein information. *Nucleic Acids Res* **43:** D204–D212.

Tremblay, P. L., Aklujkar, M., Leang, C., Nevin, K. P., and Lovley, D. (2012) A genetic system for *Geobacter metallireducens*: role of the flagellin and pilin in the reduction of Fe(III) oxide. *Environ Microbiol Rep* **4:** 82–88.

Ünal, B., Perry, V. R., Sheth, M., Gomez-Alvarez, V., Chin, K.-J., and Nüsslein, K. (2012) Trace elements affect methanogenic activity and diversity in enrichments from subsurface coal bed produced water. *Front Microbiol* **3:** 262–275.

Wickham, H. (2009) ggplot2: elegant graphics for data analysis.[WWW document]. https://CRAN.R-project.org/package=ggplot2.

Youngblut, N. D., Dell'Aringa, M., and Whitaker, R. J. (2014) Differentiation between sediment and hypolimnion methanogen communities in humic lakes. *Environ Microbiol* **16:** 1411–1423.

Zhang, T., Tremblay, P. L., Chaurasia, A. K., Smith, J. A., Bain, T. S., and Lovley, D. R. (2013) Anaerobic benzene oxidation via phenol in *Geobacter metallireducens*. *Appl Environ Microbiol* **79:** 7800–7806.

Zhang, H., Huang, T., and Chen, S. (2015) Ignored sediment fungal populations in water supply reservoirs are revealed by quantitative PCR and 454 pyrosequencing. *BMC Microbiol* **15:** 1–11.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Groundwater characteristics of the coal seam well measured at the start of the experiment (0 months).
Table S2. Overview of SSU seed sequence and protein sequence analyses.
Fig. S1. Schematic of a coal seam gas well.