International Journal of Coal Geology 160–161 (2016) 42–50

Contents lists available at ScienceDirect

International Journal of Coal Geology

journal homepage: www.elsevier.com/locate/ijcoalgeo

Revealing colonisation and biofilm formation of an adherent coal seam associated microbial community on a coal surface

Silas H.W. Vick [a,b,*], Sasha G. Tetu [b], Neil Sherwood [a], Kaydy Pinetown [a], Stephen Sestak [a], Pascal Vallotton [a], Liam D.H. Elbourne [b], Paul Greenfield [a], Errin Johnson [c,d], Deborah Barton [c], David J. Midgley [a], Ian T. Paulsen [b]

[a] Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia
[b] Department of Chemistry and Biomolecular Sciences, Macquarie University, NSW, Australia
[c] School of Biological Sciences, University of Sydney, NSW, Australia
[d] Dunn School Bioimaging Facility, Oxford University, United Kingdom

ARTICLE INFO

Article history:
Received 7 March 2016
Received in revised form 26 April 2016
Accepted 26 April 2016
Available online 29 April 2016

Keywords:
Coal seam microbiology
Geomicrobiology
Microbial community
Biofilm
Community succession

ABSTRACT

The discovery that coal seam microbial communities contribute appreciably to coal seam methane (CSM) reserves worldwide has led to an increased interest in the coal seam microbiome. While studies to date have focussed on characterising the microbial communities in a mature state, very little has been reported on the physical niche partitioning and colonisation processes of these communities on coal surfaces. Coal represents a difficult substrate for microbial characterisation using classical techniques due to in its adsorptive nature and recalcitrance to reflectance and fluorescence-based microscopy. This study presents a new technique involving culturing on specially prepared polished coal disks which allows for examination of microbes adherent to the coal surface using both molecular and microscopic approaches. Using this technique we have investigated the colonisation process of the coal surface including evidence for the involvement of a biofilm and successional changes in abundance of several community members during colonisation.

1. Introduction

Understanding how microbes convert organic matter in coal into methane (natural gas) has been the subject of increasing research effort. The research is in part aimed at generating power using lower carbon emission technologies, where coal seam methane (CSM) provides an important bridge technology to carbon-free renewables.

Microbial degradation of these compounds requires an array of enzymatic activities. In aerobic environments the degradation of the aromatic compounds is facilitated by various laccases and peroxidises (Quigley, 1993), but, in anaerobic environments different mechanisms are required (Carmona and Diaz, 2005; Fuchs et al., 2011). The initial anaerobic coal-degrading reactions are mainly limited to bacteria, and variously use nitrate, sulfate, ferric iron and even humic substances as terminal electron acceptors or 'shuttles' (Gibson and Harwood, 2002; Lovley et al., 1996; Lovley et al., 1989). These reactions proceed via a series of successive degradations of complex compounds with different microbes involved in the various stages of degradation (Kotsyurbenko, 2005; Stams, 1994). Coal may be depolymerised in a fashion similar to the anaerobic depolymerisation of lignin (Deobald, 1993) which proceeds with vanillin, vanillic acid and fatty acids as intermediates (Chen et al., 1987). Simple aromatic compounds may then be dehydroxylated and decarboxylated prior to degradation of the aromatic ring (Boll and Fuchs, 2005; Harwood et al., 1998). After ring cleavage a range of compounds are produced that can be variously fermented by bacteria to CO_2, H_2, and alcohols along with butanoates and to methane via acetoclastic archaea, or syntrophic CO_2-reducing methanogenesis by hydrogenotrophic archaeal taxa (Kotsyurbenko, 2005; Schink, 1997).

To date, studies characterising microbial communities in coal seams and their corresponding formation waters were mostly based on small scale Sanger sequencing or restriction digestion analysis of 16S rRNA gene sequence clone libraries constructed from PCR amplifications of environmental or microcosm samples (Fry et al., 2009; Green et al., 2008; Li et al., 2008; Midgley et al., 2010; Penner et al., 2010; Shimizu et al., 2007; Strapoc et al., 2008; Tang et al., 2012; Singh et al., 2011; Klein et al., 2008; Deppenmeier et al., 2002). Additionally there are a growing number of recent studies utilising next-generation sequencing technologies to perform direct 16S rRNA gene amplification and sequencing (Guo et al., 2012; Wei et al., 2013; Zhang et al., 2015; Susilawati et al., 2014; Susilawati et al., 2015; Wei et al., 2014; Raudsepp et al., 2015) and two reports of direct metagenomic sequencing of environmental DNA (Ghosh et al., 2014; Lawson et al., 2015). These studies have focussed on mature methanogenic communities past the colonisation stage and are largely concerned with how

* Corresponding author at: Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia.
E-mail address: silas.vick@csiro.au (S.H.W. Vick).

http://dx.doi.org/10.1016/j.coal.2016.04.012
0166-5162/

variations in the source, including coal, inorganic rocks and formation waters associated with a given coal seam, relate to differences in community composition.

A small number of these studies (Klein et al., 2008; Guo et al., 2012; Wei et al., 2013) have noted some structural separation between communities on coal and planktonic communities in the formation water. This raises the intriguing prospect of a distinct adherent biofilm community on surfaces and a planktonic community in formation water within coal seams.

In much of the traditional microbiological literature, there has been a tendency to visualise bacteria as being solitary, self-sufficient and antisocial, as tiny single-celled 'islands'. Increasingly, however, it is becoming clear that this is not an accurate picture of the majority of bacterial life (Hall-Stoodley et al., 2004).

Since the very beginnings of microbiology as a science, the idea of bacteria as independent 'islands' has been called into question. In 1684, Anthony van Leewenhoek described the biofilm that forms on human teeth, rendering the resident microbes protected from washing with strong vinegar. From this earliest date, it has been evident that biofilms are not simply passive assemblies, but that aggregation offered benefits, in this first case, protection.

Currently, it is clear that biofilms are dynamic, complex, non-random structures that offer a host of benefits to the organisms living therein. These include the creation of a microenvironment that is potentially advantageous in terms of avoiding the physicochemical extremes of the open environment. Biofilms also provide close contact between component members, thus supporting opportunities to engage in symbiotic and syntrophic processes. If biofilms were to form on coal surfaces, they may host organisms that liberate and transform the organic matter. These organisms, commonly referred to as the "first biters", potentially hold keys to further enhancing coal seam gas production using microbes (Schlegel et al., 2013; Mahaffey, 2012).

Visualising microorganisms in or on coal is hampered by the coal itself because it has highly adsorptive surfaces and readily binds organic dyes and other markers normally used to visualise microbes (van Krevelen, 1961; Mittal and Venkobachar, 1993). Complications also arise from coal's autofluorescence at almost all of the visible frequencies of light. Finally, the highly torturous surface of coal leads to problems in observing microbes in a single plane. In the current study, a method for visualising microbes on coal is presented and its implementation demonstrated in a pilot experiment using a coal sample from the Bowen Basin, Australia and formation water from the Sydney Basin, Australia.

2. Methods

2.1. Preparation of the coal disks

Coal was sourced from an above ground coal mine in the Rangal coal measures of the Bowen Basin, coal was collected as a single piece, wrapped in plastic on site then stored at room temperature in the laboratory before use. The coal was cored parallel to bedding using a 10 mm diameter diamond coring drill. The cores were then ground perpendicular to bedding, to a thickness of 2.5 mm using silicon carbide abrasive papers on a rotating plate. One disk face was then polished on rotating laps using aluminium oxide and colloidal silica polishing media. Water was the sole lubricant used for all cutting, coring, grinding and polishing. Resultant coal disks had sufficient integrity to be wired, manipulated for culturing and prepared for SEM imaging without breakage or loss of the polished surface.

2.2. Source of inoculum

Formation water was sampled from the liquid-gas separator of a dual seam CSG well (MP09) located at Menangle Park (34°6′S 150°44′ E), NSW Australia. The water was sourced from the Bulli and Balgownie seams of the Illawarra coal measures, intersected at 666 m and 683 m depths respectively. The formation water was incubated anoxically at 30 °C for approximately 6 months prior to use. Chemical analysis of the water (Table 1) was performed by the National Measurement Institute (NMI), Sydney Australia.

2.3. Community profiling of the source of inoculum

In order to understand which microbes occur in a given sample, the microbial community was profiled by examining DNA sequences (16S rRNA). The 16S rRNA sequence contains variable and conserved regions and is thus a useful marker for determining microbial identity.

Six hundred milliliters of formation water was filtered through 0.2 μm polyvinylidene fluoride (PVDF) filter disks (Merck Millipore, Bayswater, Victoria, Australia). The filter disks were then segmented using a sterile scalpel and filter disk segments were subjected to DNA extractions. DNA extractions of the microorganisms on the segmented filter disks were performed with a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.) as per manufacturer's instructions. 16S rRNA amplicon sequencing was performed on the DNA extraction using the Illumina MiSeq platform to generate 300 bp paired-end reads and 20,000 reads/sample. Library preparation, amplification and sequencing was performed by Mr DNA (Molecular Research LP, Texas, USA) using the 515F and 806R universal 16S rRNA primer sequences which match conserved regions flanking a ~300 base pair (bp) variable region of the 16S rRNA gene (Caporaso et al., 2012).

2.4. Coal disk incubation

Two coal disks attached to a 0.5 mm diameter nickel wire were held vertically in 160 ml borosilicate glass serum vials and 20 ml of (M9) minimal salts medium containing 100 mg/l NH_4Cl, 400 mg/l K_2HPO_4, 100 mg/l $MgCl_2$, 0.0001% resazurin, 1 ml/l SL-10 trace element solution, 250 mg/l Na_2S, 200 mg/l cysteine HCl and 1 ml/l Wolins vitamin solution (Wolin et al., 1963) was added to the vials to submerge the disks under anoxic conditions (headspace 95% Ar, 5% H_2). Vials were sealed with butyl rubber stoppers and incubated in the dark at 30 °C without shaking. Vials were removed for visualisation at 0 (pre-inoculation), 4, 8, 16, 24, 34 and 48 days post-inoculation.

Table 1
Physicochemical characteristics of the MP09 formation water.

Test	Measured value[a]
pH	8.57
Electrical conductivity at 25 °C	13,000 μS/cm
Hydroxide alkalinity as $CaCO_3$	<1
Carbonate alkalinity as $CaCO_3$	485
Bicarbonate alkalinity as $CaCO_3$	7150
Total alkalinity as $CaCO_3$	7640
Silicon as SiO_2	22.6
Sulfur as S	<2
Sulfate as SO_4 - turbidimetric	<10
Chloride	634
Magnesium	5
Sodium	3650
Potassium	20
Iron	0.99
Barium	19.6
Lithium	8.94
Strontium	6.05
Bromine	1.72
Ferrous iron	0.08
Nitrite as N	<0.05
Nitrate as N	<0.05
Total nitrogen as N	7.8
Total phosphorus as P	0.1

[a] Measurements are in mg/l unless otherwise stated.