A metaproteomic approach for identifying proteins in anaerobic bioreactors converting coal to methane

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Abstract

To understand the processes involved in bioconversion of coal to methane, a metaproteomic approach was taken to identify proteins in microcosms containing coal, standard medium and an adapted microbial community. Concentrated and dialyzed protein samples were subjected to further cleanup and trypsin digestion followed by mass spectrometric analysis. Searching the generated peaklists against domains of bacteria, archaea and fungi revealed 152 ± 1.4, 96.5 ± 2.1 and 38 ± 1.4 protein families, respectively. Proteins associated with bacteria were distributed among transporter and membrane proteins (33.1%), cellular metabolism (28.5%), substrate utilization/conversion (7.3%), oxidative stress (5.3%), cell movement (3.3%) and hypothetical proteins (22.5%). Among the total archaea proteins, 37.8% were for substrate utilization related to methane production, 27.6% were for cellular metabolism, 6.1% responded to stress, 5.1% were transporter and membrane proteins and 23.5% were those with unknown functions. Proteins produced by fungi fell in two groups: cell metabolisms (23.5%) and hypothetical proteins (54.3%). Based on key enzymes identified, a pathway for methanogenesis in the tested samples was proposed. This pathway illustrated methane production from four starting compounds, acetate, formate, methanol and CO2. The proposed pathway will serve as a solid foundation for future effort aiming to increase methane yield from coal.

Keywords: Coal, Methane, Microbial consortium, Extracellular proteins, LC/MS

1. Introduction

During recent years, considering the environmental drawbacks of generating electricity from coal combustion, converting coal to methane through biological processes has attracted significant attention (Fallgren et al., 2013; Wei et al., 2014). As a result, formation water samples collected from different coal seams have been evaluated in terms of the potential for producing methane. Several studies through constructing clone libraries or next generation pyrosequencing have been conducted for coals from the Powder River Basin (Ayers, 2002; Flores et al., 2008; Green et al., 2008; Ulrich and Bower, 2008), the San Juan Basin (Scott et al., 1994), the Illinois basin (Strapoč et al., 2008), the Indio formation (Jones et al., 2010), the Alberta coalbeds in western Canada (Penner et al., 2010), the Jiujiang Formation in the Jingmen-Danyang basin in Hubei, China (Wei et al., 2014), the south Sydney Basin (Faiz and Hendry, 2006) and others listed in the review (Strapoč et al., 2011). As a consequence, communities of fermentative and acetogenic bacteria and methane-releasing archaea have been identified in different subsurface environments. However, although the microbial distribution in a given place is known, the microbial functionality remains largely unclear.

Metabolic activities of a microbial community can be characterized by isotope analysis or through the analysis of methane production. From the perspective of molecular biology, cellular activities can also be revealed by the analysis of: 1) transcripts or metatranscriptome, the collective mRNA from all microorganisms in an ecosystem and 2) proteins or metaproteome, the collective proteins from all microbial species present in an ecosystem (Stokke et al., 2012). While the former provides insights into gene expression and activity, not all expressed genes will participate in certain pathways due to additional levels of cellular localization and regulation which occur at the protein level (Vanwonterghem et al., 2014). Thus, only results from the metaproteome study can give direct evidence of cellular metabolic activities at molecular levels.

Benefited from the rapid development of mass spectrum instrumentation and bioinformatics software, metaproteomic analysis has been performed for various samples, such as: a mesophilic biogas-producing community fermenting straw and hay (Hanreich et al., 2013), a complex microbial community producing methane from agricultural waste and energy crops (Heyer et al., 2013), a microbiota in the phyllosphere and rhizosphere or rice (Knief et al., 2011), a microbial community from an anaerobic industrial-like wastewater treatment bioreactor (Abram et al., 2011), proteins present in the extracellular...
polymeric substances of active sludge flocs (Park et al., 2008), and an ANME (anaerobic methanotrophic archaea) community in marine cold seep sediments (Stokke et al., 2012). For communities degrading coal to methane, however, no such investigations have ever been conducted.

Recently, an original microbial community collected from a coalbed methane (CBM) well in the Illinois basin and an adapted consortium developed from it were studied through next-generation sequencing. Both the original and the adapted consortium contained bacterial and archaeal species and produced methane from coal in a laboratory setting (Zhang et al., 2015). To understand the functionality of the microbial community and the pathways leading to methane from coal, we aimed to identify proteins in anaerobic microcosms designed for bioconversion of coal to methane. Instead of using the traditional 2-dimensional gel electrophoresis which is likely to result in biased results towards the most abundant proteins (Abram et al., 2011), we adopted the state-of-the-art proteomic approach to separate and identify target proteins. Based on the proteins detected, a pathway for methanogenesis was proposed here.

2. Materials and methods

2.1. Coal samples

Coal samples used in this study were the same as those investigated in another work (Zhang et al., 2015). Briefly, chunks of high volatile B bituminous coals were collected from Herrin Seam (No. 6) of the Illinois basin. The coals were ground and fragments that were retained between 40 and 100 mesh (0.15–0.425 mm) screen was stored in Ziploc bags and maintained in a humidity chamber to avoid water loss.

2.2. Methane production

A maintenance culture of the adapted consortium initially developed from the CBM community was established in our laboratory. To set up the duplicate microcosms, which were 100 mL serum bottles, the maintenance culture serving as the inoculum was added in a volume of 10% of the final total volume to 10 g fresh coal in 45 mL of a standard medium (Bonin and Boone, 2006). The two bottles were then closed with a butyl rubber stopper, sealed by an aluminum crimp and kept in dark at 28 °C.

To understand the bio-conversion process better, methane yields from different controls were also evaluated. These controls included: 1) both coal and the inoculum autoclaved. This was to test whether microbial contamination took place during microcosm cultivation; 2) coal with autoclaved inoculum. This was to evaluate whether microorganisms associated with coal can produce methane; and 3) the inoculum and medium only (without coal). This was to determine whether the consortium can generate methane from the supplemented medium. As stated above, for each condition, two replicates were established. All microcosms were maintained at 28 °C in the dark. At days 10, 20 and 30, samples from the headspace in each serum bottle were analyzed by gas chromatography (GC) as described previously (Zhang et al., 2015).

2.3. Protein identification

2.3.1. Sample preparation

Immediately after day 30, the microcosms were frozen at −20 °C. Upon use, the entire content in each microcosm was allowed to thaw first, followed by transferring to centrifugation tubes. The liquid portion after centrifuging the entire content at 4,000 g for 15 min was further
vacuum-filtered through 0.2 μm sterile filters. To the collected filtrate, a volume of 10 μl of Halrt™ Protease Inhibitor Single-Use Cocktail EDTA-free (Pierce Biotechnology, Rockford, IL, USA) was added. The filtrate was then processed through Pierce Concentrators (9 K MWCO, 20 mL, Pierce) following the manufacturer recommended procedures. The concentrated protein samples were further dialyzed against distilled and deionized water three times. The dialyzed and concentrated samples were supplemented with 10 μl Protease Inhibitor to prevent protein degradation. Protein concentrations of these final samples were measured through using a BCA Protein Assay kit (Pierce) according to the manufacturer’s protocol.

To prepare samples for protein identification, the samples were further cleaned by using Perfect Focus (G-Biosciences, St. Louis, MO, USA) according to the manufacturer’s recommendation. Cleaned samples were digested with MSG-Trypsin (G-Biosciences) in 25 mM ammonium bicarbonate at a ratio of 1:10–1:50 (w/w) using a CEM Discover Microwave Digestor (Mathews, NC, USA) at 55 °C and maximum power of 60 W for 30 min. Digested peptides were lyophilized and re-suspended in 5% acetonitrile plus 0.1% formic acid.

2.3.2. UPLC/MS

Ultra performance liquid chromatography (UPLC) was performed using a Thermo Dionex Ultimate RSLC3000 operating in nano mode at 300 nL/min with a gradient from water containing 0.1% formic acid to 100% acetonitrile + 0.1% formic acid in 200 min. The trap column used was a Thermo Acclaim PepMap 100 (100 μm × 2 cm) and the analytical column was a Thermo Acclaim PepMap RSLC (75 μm × 15 cm). The mass spectrometer used was the highly sensitive Thermo LTQ Velos Pro MS.

2.3.3. Data analysis

Xcalibur raw files were converted by Mascot Distiller into peaklists that were submitted to an in-house Mascot Server and searched against specific NCBI-NR protein databases for archaea, bacteria and fungi.

3. Results and discussions

3.1. Methane production

Headspace gas analysis of different microcosms revealed that (Fig. 1): 1) no CO₂ and CH₄ were observed in serum bottles with autoclaved coal and inoculum, which indicated that no microbial contamination took place during the 30-day cultivation period and autoclave adequately deactivated any microbial activities within coal and the inoculum, which was the acclimated microbial consortium; 2) increased CO₂, but no CH₄ was released with time from microcosms containing coal and the autoclaved inoculum, which demonstrated that the microbial strains associated with coal samples could degrade coal to CO₂. But these cells were not able to produce methane even though archaea strains similar to Methanobrevibacter sp. were present (Zhang et al., 2015); 3) increased CO₂, but no CH₄ was detected in serum bottles with the inoculum and medium only. For these setups, no coal was provided. Thus, the released CO₂ was from organic carbon in the medium provided. Grown on this nutrient solution, however, the adapted microbial consortium did not produce any CH₄ though species close to Methanobacterium bryantii and Methanobrevibacter arborophilus were identified (Zhang et al., 2015). Therefore, all methane observed in our experiments was from coal itself but not from any nutrients provided.

Nutrient, such as yeast extract was speculated to be a source for methane production during the first 72 h of cultivation (Green et al., 2008). But in this study, we proved that yeast extract and peptone did not lead to methane release; and 5) with the presence of coal and the inoculum, the standard medium gave a methane yield of 120.0 ft³/ton in 30 days, which was similar to 111 ft³/ton detected previously in 20 days (Zhang et al., 2015). These calculations assumed that the powdered coal samples we used were uniform in composition. Although the overall yield was close, the methane production rate of 4.0 ft³/ton/day was lower than 5.6 ft³/ton/day we observed before. The reason was that, for this experiment, we used coals that had particle sizes between 40 and 100 mesh, coarser than those used in previous experiments, which were <40 mesh. This is in agreement with previous report that finer coals lead to higher methane production rate even for lignite (Harding et al., 1993).

3.2. Protein identification

The conventional procedure for identifying proteins in metaproteomes generally has two steps: protein separation by 2-D polyacrylamide gel electrophoresis (PAGE) and protein identification by LC/MS/MS. Although this approach has its own advantages, in particular, related to allowing visual comparison of protein up- or down regulation, it suffers from the fact that only abundant proteins with high spot density on the gel can be accurately excised and identified. In addition to this drawback, mass spectrometer with low sensitivity hinders proper protein detection. As a result, some successful metaproteome studies have reported identification of very limited number of proteins, such as, 17 (Hanreich et al., 2012; Heyer et al., 2013), 18 (Abram et al., 2011) and 36 (Hanreich et al., 2013). In the current study, however, the improved ion optics of the Thermo LTQ Velos Pro MS together with the nano-UPLC separation enabled us to achieve superior sensitivity and resolution for protein identification without going through gel separation. The great capability of this MS has been demonstrated by identifying 158 proteins in chicken egg white proteome (Mann and Mann, 2011) and other numerous studies.

For this investigation, processed protein samples from three sets of microcosms were analyzed by UPLC/MS. These three sets were: 1) two replicates which contained coal, the standard medium and the inoculum; 2) two microcosms which comprised the standard medium #1 + #2, not in #3, #4, and #5.

### Table 1
Summary of protein identification from different samples.

<table>
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<th>Samples</th>
<th>Bacteria</th>
<th>Archaea</th>
<th>Fungi</th>
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<td>Average ± standard deviation</td>
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<td>#2</td>
<td>204</td>
<td>153</td>
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<tr>
<td>#3</td>
<td>311</td>
<td>246</td>
<td>240.5 ± 7.8</td>
</tr>
<tr>
<td>#4</td>
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<td>235</td>
<td></td>
</tr>
<tr>
<td>#5</td>
<td>150</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>In #1 + #2, not in #3, #4, and #5</td>
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<td></td>
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</tbody>
</table>

#1 and #2: Replicates of microcosms containing coal, the inoculum and the standard medium.

#3 and #4: Replicates of microcosms containing the inoculum and the standard medium, but without coal.

#5: One replicate containing coal and the standard medium, but without the inoculum. Another replicate was lost during sample preparation.
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<th>Accession</th>
<th>Score</th>
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### Table 3

List of statistically valid proteins identical to those in archaea. Highlighted are those that are present in samples #1 and #2, but not in samples #3, #4 and #5.

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<th>Accession</th>
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</tr>
</tbody>
</table>

(continued on next page)
and the inoculum, but without coal; and 3) one replicate which included the coal samples and the standard medium, but without the inoculum. For the third group, one replicate was lost during sample preparation. As shown in Table 1, a large number of proteins with confidence level of 95% (p < 0.05) were detected in all five samples. Using a Mascot cutoff score of 43, all observed proteins were divided into two

| Table 1 | (continued) |
|-----------------|------------------|-----------------|-----------------|------------------|------------------|
| 44 | 35 | gi|499329625 | 84 | 7984 | 1.77 | Deoxyribonuclease | Methanosarcina acitivorans |
| 45 | 22 | gi|17380265 | 118 | 22967 | 0.73 | Proteasome subunit alpha | Methanosarcina thermophila |
| 46 | 30 | gi|499342822 | 102 | 15502 | 0.71 | Pyridoxamine 5′–phosphate oxidase | Methanosarcina mazei |
| 47 | 21 | gi|49037782 | 120 | 27138 | 0.6 | Proteasome subunit alpha | Methanosarcina thermophila |
| 48 | 26 | gi|499330993 | 107 | 9538 | 0.54 | Effector protein | Methanosarcina acitivorans |
| 49 | 29 | gi|499345514 | 103 | 13262 | 0.37 | 4-carboxymuconolactone decarboxylase | Methanosarcina mazei |
| 50 | 14 | gi|499333059 | 155 | 14789 | 0.33 | Cupin | Methanosarcina acitivorans |
| 51 | 51 | gi|504371269 | 58 | 16609 | 0.29 | associated domain–containing protein | Fervidicoccus fontis |
| 52 | 48 | gi|499345198 | 61 | 17072 | 0.28 | Peptidylprolyl isomerase | Methanosarcina mazei |
| 53 | 61 | gi|499168240 | 51 | 17740 | 0.27 | Aspartate carbamoyltransferase | Aeropyrum pernix |
| 54 | 19 | gi|499626578 | 127 | 37655 | 0.25 | NADP−dependent alcohol dehydrogenase | Methanosarcina barkeri |
| 55 | 86 | gi|499490599 | 45 | 23940 | 0.19 | 50S ribosomal protein L1 | Picrophilus torridus |
| 56 | 34 | gi|511307100 | 87 | 48911 | 0.19 | Glutamate dehydrogenase GdhA | Methanobrevibacter sp. AbM4 |
| 57 | 84 | gi|495251456 | 45 | 26401 | 0.17 | Cytochrome C | Halobadattus pauchalophilus |
| 58 | 15 | gi|1199638 | 149 | 63804 | 0.14 | A1AO H+ ATPase, subunit A | Methanosarcina mazei Go1 |
| 59 | 53 | gi|46396470 | 55 | 32230 | 0.14 | Pyridoxal biosynthesis lyase PdxS | Methanosarcina acitivorans |
| 60 | 67 | gi|494645170 | 49 | 38444 | 0.12 | SAM−dependent methyltransferase | Candidatus Nitrosocarchaeum limnia |
| 61 | 90 | gi|499343317 | 45 | 37687 | 0.12 | Endonuclease | Methanosarcina mazei |
| 62 | 76 | gi|50349780 | 47 | 37850 | 0.12 | Methylthioribose−1−phosphate isomerase | Archaeoglobus veneficus |
| 63 | 70 | gi|499316941 | 48 | 39075 | 0.11 | Endonuclease | Pyrobaclum aerophilum |
| 64 | 95 | gi|498405725 | 44 | 39785 | 0.11 | UDP−N−acytylglosamine 2− epimerase | Haloflexa larseni |
| 65 | 80 | gi|493478001 | 47 | 48340 | 0.09 | IS1341−type transposase (TCE42) | Natronimena versiforme |
| 66 | 87 | gi|505313913 | 49 | 49042 | 0.09 | Nucleotide sugar dehydrogenase | Natronococcus occultus |
| 67 | 82 | gi|50504812 | 46 | 58596 | 0.08 | Dihydroxyacid dehydratase | Thermoplasmatales archaeon BRNA1 |
| 68 | 66 | gi|505222968 | 49 | 62963 | 0.07 | Flagella biogenesis protein FlA1 | Natronomonas moolapensis |
| 69 | 74 | gi|339756697 | 48 | 62119 | 0.07 | Arginyl−tRNA synthetase | Candidatus Nanosalinarum |
| 70 | 52 | gi|519065234 | 55 | 63573 | 0.07 | NADH−quinone oxidoreductase subunit C | Halarchaeum acidiphilum |

| Stress response (6) |
|------------------|------------------|------------------|------------------|------------------|------------------|
| 38 | 71 | gi|499627762 | 48 | 23931 | 0.19 | Superoxide dismutase | Methanosarcina barkeri |
| 39 | 46 | gi|499344888 | 62 | 24611 | 0.19 | Superoxide dismutase | Methanosarcina mazei |
| 40 | 94 | gi|494102747 | 44 | 16407 | 0.29 | Universal stress protein | Methanotorris formicicus |
| 41 | 18 | gi|499333494 | 131 | 16370 | 0.29 | Universal stress protein | Methanosarcina acitivorans |
| 42 | 31 | gi|499332655 | 101 | 10442 | 2.27 | Thioredoxin | Methanosarcina acitivorans |
| 43 | 50 | gi|499625148 | 58 | 17859 | 0.26 | Heat−shock protein | Methanosarcina barkeri |

| Transporter and membrane proteins (5) |
|------------------|------------------|------------------|------------------|------------------|------------------|
| 71 | 4 | gi|499333555 | 419 | 50365 | 1.33 | V−type ATP synthase subunit B | Methanosarcina acitivorans |
| 72 | 6 | gi|393715204 | 273 | 31948 | 0.94 | S−Layer (Ma0829) Protein | Methanosarcina Acitivorans |
| 73 | 36 | gi|339757710 | 80 | 27518 | 0.17 | ABC−type Fe3+−hydroxamate transport system, periplasmic | Candidatus Nanosalinarum sp. J07AB43 |
| 74 | 45 | gi|493940982 | 62 | 39158 | 0.11 | Basic membrane protein | Halosimpex carlsbadense |
| 75 | 39 | gi|499180850 | 68 | 43703 | 0.1 | ABC transporter substrate−binding protein | Archaeoglobus fulgidus |
groups: statistically valid ($\geq 43$) and statistically uncertain ($< 43$) groups. Searching the peaklists against domain of bacteria, archaea and fungi revealed the presence of 152 $\pm$ 1.4, 96.5 $\pm$ 2.1 and 38 $\pm$ 1.4 protein families, respectively, for the first set of samples. Regarding the second set, the numbers of protein families were 240.5 $\pm$ 7.8 for bacteria, 64 $\pm$ 2.1 for archaea and 38 $\pm$ 1.4 for fungi. The third set contained 115 proteins, 37 from archaea and 10 fungal proteins that were present in all three sets. Comparing all three sets, there were 39 bacterial proteins related to bacteria, 24 proteins belonged to archaea and 19 proteins related to fungi. Comparing the three sets, there were 8 proteins that were from bacteria, archaea and fungi, respectively, in the first set, but not in the second and third group. Since methanogenesis is a key process for growth and for producing methane, it could be deduced that the bacterial cells could utilize methanogens as the energy source for growth and for producing methane. The fact that 33.1% of bacterial proteins were related to substrate binding and transport demonstrated that bacterial cells devoted significant amount of energy and effort trying to grab whatever was available in the microcosms.

The second category was cellular metabolism. Among 43 of this group, glutamate dehydrogenase was the most abundant with an emPAI of 1.38. This enzyme converts glutamate to oxoglutarate while releasing NH$_4^+$ through the reaction (Buckel, 2001). It is unknown at this stage whether the presence of five different families of this enzyme in the day-30 microcosms was related to nitrogen deficiency. The third group included 11 proteins that might be involved in substrate utilization and conversion. From the five representative enzymes: iron hydrogenase, glucokinase, rhamnulokinase, glycoside hydrolase and sulfite reductase, it could be deduced that the bacterial cells could utilize glucose, rhamnose, mixed sugars and sulfite for growth and for producing hydrogen. This group also had one family of methyl-CoM reductase (Mcr) which is the enzyme responsible for converting methyl-CoM to methane. Abundance of this protein family is fairly high with an emPAI of 0.45. Since steps and enzymes unique to the aceticlastic path- way are widely distributed in the domain of bacteria (Ferry, 2010),...
detection of this protein was not surprising. However, since this enzyme matched one in an uncultured bacterium (gi|167541422), no more details could be described here.

The fourth group was eight protein families that were responsive to oxidative stress. These proteins included: rubrerythrin (emPAI = 1.29) which has been proposed as a scavenger of oxygen radicals responding to oxidative stress (Lehmann et al., 1996); superoxide dismutase and peroxidase which reduces superoxide and peroxide, respectively and are described as main detoxification systems in bacteria for oxygen resistance and reduction (Zhang et al., 2006). The presence of these oxidoreductases corresponded well to transient exposure to oxygen during microcosm setup. The fifth group comprised five protein families that were related to cell movement. Flagella contribute to cell movement through chemotaxis and adhesion to host surfaces. Flagellin is the structural protein that forms the major portion of flagellar filaments (Ramos et al., 2004). The identification of five different families of flagelin proteins demonstrated that bacterial cells spent quite amount of energy in producing flagella and getting them moved to places where substrates might be available. The last group contained all of those 34 hypothetical proteins whose functions were not known at this point. These proteins might have important roles in the coal-to-methane pathway. But due to lack of studies and limited information, no specific names could be given.

Searching the generated peaklists from sample #1 against archaea domain revealed 98 protein families (Table 3). Similarly, these proteins were categorized into different groups. The first

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<td>53</td>
<td>132725</td>
<td>0.03</td>
<td>Putative histidine kinase M232p</td>
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<tr>
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<td>gi</td>
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<td>56</td>
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<td>0.02</td>
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<td>32</td>
<td>gi</td>
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<td>54</td>
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<td>0.02</td>
<td>Probable Glycogen debranching enzyme</td>
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<tr>
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<td>46</td>
<td>gi</td>
<td>667663171</td>
<td>44</td>
<td>229441</td>
<td>0.02</td>
<td>Pol–like protein</td>
</tr>
</tbody>
</table>
A group of 37 proteins or 38% of the total was tied to methane production. The most abundant protein (emPAI = 5.09) was methylcoenzyme M reductase (Mcr) with eight different families. These proteins were close to those in Methanosarcina mazei, Methanosarcina acetivorans, Methanoculleus marisnigri, Methanosaeta thermophile, Methanoplanus limicola and an uncultured archaeon. This enzyme converts methyl coenzyme M and coenzyme B to methane and heterodisulfide, the last step in methane formation from CO₂, acetate and methanol (Scheller et al., 2013). The abundance of these proteins demonstrated that methane production was very active in the studied sample. Regarding the aceticlastic pathway from acetate to methane (Hanreich et al., 2012), three enzymes, acetate kinase, phosphotransacetylase and acetyl-CoA decarboxylase synthase were detected. In terms of CO₂ reduction, three out of the six enzymes dedicated for CO₂ reduction were observed. These enzymes were: formyl-methanofuran dehydrogenase (Fmd, the first enzyme in CO₂ reduction (Sakai et al., 2011)); methylene tetrahydromethanopterin (H₄MPT) dehydrogenase (Mtd; the fourth enzyme in CO₂ reduction); and methylene tetrahydromethanopterin (H₄MPT) reductase (Mer, the fifth enzyme in CO₂ reduction). In addition, four different protein families of methanol-5-hydroxybenzimidazolylcobamide methyltransferase were identified. Two proteins (gi|499331135, gi|12751300) resembled those in Methanosaeta thermophile and the other two (gi|499625345, gi|499627806) were similar to those in Methanosarcina barkeri. These proteins catalyze the transfer of a methyl group from methanol to a methanol-specific corrinoid protein (Mta) and are involved
versal stress proteins. The fourth group contained plant fragments in the rumen of cattle and sheep on were produced at low levels according to the low emPAI values.

Those belonging to bacteria and archaea, all of these fungal proteins methyltransferase, ATPase, surface glycoprotein, histidine kinase, etc. were dedicated for substrate binding and transport. These proteins in-

cluded six proteins that appeared to be responding to oxidative stress. Among the six, thioredoxin had an emPAI of 2.27. This enzyme is known to reduce hydrogen peroxide and certain radicals. Similarly to those belonging to bacteria and archaea, all of these fungal proteins were produced at low levels according to the low emPAI values.

Anaerobic fungi with large populations have been shown to colonize plant fragments in the rumen of cattle and sheep on fibrous diets (Bauchop, 1981; Orpin and Joblin, 1997) and in anaerobic digesters treating organic wastes (Schnürer and Schnürer, 2006). However, although metaproteomic studies have been conducted on plant-based feedstocks (Hanreich et al., 2013) and agricultural wastes (Heyer et al., 2013) as detailed above, no studies have reported the presence of fungal proteins. The low abundance of fungal proteins as demonstrated in this study might explain why fungal proteins have never been demonstrated before in anaerobic digesters. Adding to these low levels of proteins, the traditional way of 2-D PAGE only enables proteins with high density to be picked and identified (Abram et al., 2011; Hanreich et al., 2012, 2013). Thus, assisted by the high resolution LC/MS, this is the first study to report the presence of fungal proteins in anaerobic bioreactors converting coal to methane. This is in agreement with our microscopic observation that filamentous fungal species did exist in the microcosms. Based on DNA sequencing reported in our previous study, fungal stains came from the coal samples that we have been using and were not present in the original microbial community collected from a coal-bed methane (CBM) well (Zhang et al., 2015). If other coal samples are used in similar studies, fungal strains and related proteins may not be present. Regarding this study, although 46 proteins were related to fungi, fungal enzymes specific to coal hydrolysis, fermentation and methane production were not obviously detected in sample #1. Thus, the roles and functions of the fungal strains in the microbial community cannot be elucidated here.

3.3. Pathway from coal to methane

Over the years, several pathways based upon identified proteins have been proposed for methane formation from different substrates, for example, synthetic glucose-based wastewater (Abram et al., 2011) and beet and rye silage (Hanreich et al., 2012). With regard to coal, one pathway was proposed based on microorganisms identified through constructed clone libraries (Strapac et al., 2008). This pathway mainly described the coal fragmentation part and indicated that aromatic compounds, such as: polyaromatic hydrocarbons (PAHs), monoaromatic carboxylic acids and ketones were the intermediates from complex coal macromolecules. In another study, however, PAHs and ketones were not observed. Instead, single-ring aromatics, long-chain alkanes and long-chain fatty acids accumulated during the first 39 days of the 78-day study (Jones et al., 2010). This difference could be explained by examining bacterial species in the two different communities. In Strapac’s study, bacteria at the phylum level, such as Spirochaetes, Bacteroidetes, Firmicutes were identified. In Jones’ study, the dominant bacteria (56%) had 99% sequence similarity to Proteobacteria with 43% of the clones similar to Betaproteobacteria and 13% identical to Gammaproteobacteria. Regarding bacterial populations in our tested samples, Proteobacteria, Firmicutes and Bacteroidetes were 57.2%, 33.6%, and 6.7%, respectively (Zhang et al., 2015). Thus, considering the similarities of microorganisms between reported studies and this one, it is reasonable to assume that the intermediate products from coal hydrolysis in our microcosms should be similar to those reported by the two studies. However, detailed chemical studies on identifying coal degradation products are needed to prove this assumption.

As discussed above, by taking advantage of the state-of-the-art highly sensitive LC/MS, we have identified the highest number of protein families besides novel fungal proteins. Identification of these proteins allowed us to propose a pathway from coal to methane, in particular, steps involved in methanogenesis. As shown in Fig. 2, key enzymes responsible for converting acetate, CO2, formate and methanol to methane have been identified in the studied samples. Specific to the acetilic pathway, one methyltransferase (gi|499329835) that was close to that in M. acetivorans was detected. But it is unclear whether this enzyme can catalyze the step from 5-methyl-tetrahydrofolate to methyl-CoM

Fig. 2. A proposed pathway from acetate, CO2, methanol and formate to methane. Proteins identified in this study were in bold. Starting compounds were in green and methane was in red. Abbreviations: Fdh, formate dehydrogenase; Fmd, formylmethanofuran dehydrogenase; Frt, formylmethanofuran:H4MPT formyltransferase; Mch, methenyl-H4MPT cyclodrolase; Mtd, F420-dependent methylene-H4MPT dehydrogenase; Mer, methy-

lene-H4MPT reductase; Mtr, methyl-H4MPT: coenzyme M methyltransferase; Mcr, methy-

tyl-coenzyme M reductase; Ack: acetate kinase; Pta: phosphotransacetylase; ACDS: acetyl carboxylase synthetase; MT: methanol-5-hydroxymethylazoxovalomide methyl-

transferase; Mtr-H4SPT: methyl-H4SPT: coenzyme M methyltransferase. (For interpreta-

tion of the references to color in this figure legend, the reader is referred to the web version of this article.)
of this pathway will certainly assist future effort in optimizing methane yield from coal.

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