

# Keystone Microorganisms Regulate the Methanogenic Potential in Coals with Different Coal Ranks

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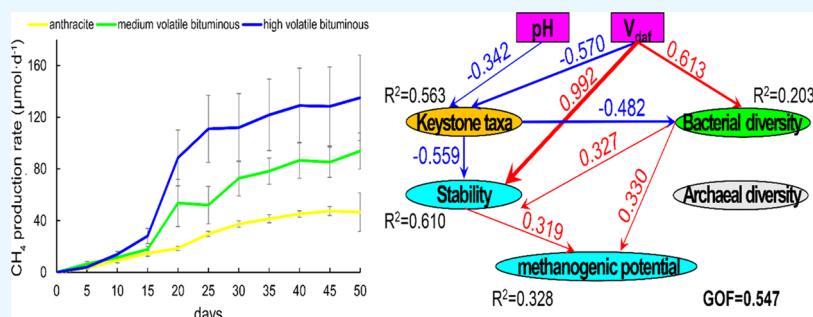
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**ABSTRACT:** Microorganisms are the core drivers of coal biogeochemistry and are closely related to the formation of coalbed methane. However, it remains poorly understood about the network relationship and stability of microbial communities in coals with different ranks. In this study, a high-throughput sequencing data set was analyzed to understand the microbial co-occurrence network in coals with different ranks including anthracite, medium-volatile bituminous, and high-volatile bituminous. The results showed similar topological properties for the microbial networks among coals with different ranks, but a great difference was found in the microbial composition in different large modules among coals with different ranks, and these three networks had three, four, and four large modules with seven, nine, and nine phyla, respectively. Among these networks, a total of 46 keystone taxa were identified in large modules, and these keystone taxa were different in coals with different ranks. Bacteria dominated the keystone taxa in the microbial network, and these bacterial keystone taxa mainly belonged to phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. Besides, the removal of the key microbial data could reduce the community stability of microbial communities in bituminous coals. A partial least-squares path model further showed that these bacterial keystone taxa indirectly affected methanogenic potential by maintaining the microbial community stability and bacterial diversity. In summary, these results showed that keystone taxa played an important role in determining the community diversity, maintaining the microbial community stability, and controlling the methanogenic potential, which is of great significance for understanding the microbial ecology and the geochemical cycle of coal seams.

## 1. INTRODUCTION

Coal is the most vital fossil fuel on earth,<sup>1,2</sup> and its formation is driven by geological events,<sup>3</sup> geologic settings,<sup>4</sup> and microbial metabolisms.<sup>5</sup> Among them, microbes are the predominant form of life in the subsurface ecosystem including coals and play vital roles in biogeochemical cycles,<sup>1</sup> which accompanied the evolution of coals over tens to hundreds of millions of years.<sup>6</sup>

Microbial activities run through the whole process from humus deposition to anthracite formation,<sup>6</sup> and are closely related to the formation of coalbed methane (CBM). Previous studies found that some organic substances in coals were degraded into methane following a quasi-step-by-step biodegradation process.<sup>1,6</sup> The macromolecular substances of coals and/or peats are first degraded into single molecules and oligomers by hydrolysis and fermentation bacteria, and then some intermediate products are generated by different

acidifying bacteria, acetic acid-producing bacteria, and hydrogen-producing bacteria. The resulting product formed generates methane under the action of methanogens. However, these studies mainly monitored the biomarkers in coals, and the interaction between these microorganisms has received extensive attention<sup>7,8</sup> merely in recent years. In addition, whether such an interaction between microorganisms can affect the methanogenic potential of coals remains unknown.

The interactions among microorganisms mainly include symbiosis, competition, parasitism, and predation.<sup>9</sup> Microbial

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networks can be widely used to study microbial interactions and their responses to environmental changes.<sup>10</sup> Strong interspecific associations among microbes in a community could provide resistance against various environmental changes<sup>11</sup> and enhance the biogeochemical processes of the ecosystem. The habitats in coal seams are quite diverse in different coal ranks (different coal-forming ages), which possess different microbial diversities.<sup>5</sup> Therefore, different coal ranks may cause changes in the microbial interactions that may affect the biogeochemical processes in coal seams (including methanogenesis). Such information is of great significance for coalbed methane generation.

Furthermore, cohesion could be used to characterize community stability.<sup>12</sup> The community stability usually has close competition and predation relationships, generally showing negative interaction or negative cohesion, and a high proportion of negative correlation/negative cohesion within a community is closely related to the community stability.<sup>13</sup> The interspecific competition of these microorganisms must also have a certain relationship with biological CBM. For example, a large amount of seawater sulfate diffuses into the bottom peat and is reduced to H<sub>2</sub>S, S, and polysulfides by microorganisms.<sup>14</sup> The release of H<sub>2</sub>S due to sulfate reduction is detrimental to the methanogenesis process during coal biodegradation.<sup>15</sup> The process of anaerobic fermentation of coal seams may also be affected by degraded intermediates and final products (such as sulfides), which at high concentrations affect pH, disrupt cell membranes, prevent protein synthesis, alter hydrogen partial pressure, reduce the bioavailability of trace elements, and hinder mass transfer, thereby disrupting the anaerobic degradation chain.<sup>16</sup> Among these inhibitory compounds, sulfide is formed by microbial reduction of sulfate and degradation of sulfur-containing organic matter under anaerobic conditions, and microorganisms involved in sulfate reduction can compete with other anaerobic taxa, especially methanogenic archaea in an environment with low redox potential.<sup>16,17</sup> In coal seams, some keystone taxa with special functions play a key role in maintaining the stability of network relationships. For example, the removal of these keystone taxa could lead to the collapse of microbial networks and functions,<sup>18</sup> and these keystone taxa may be maintaining the network structure and assembly.<sup>19</sup> Therefore, it is of great importance to analyze keystone taxa and understand the relationship of these taxa for community stability and methanogenic potential.

In summary, the different developmental stages of coal (or different coal ranks) may potentially affect the interspecific relationships and keystone microorganisms, and it is of great importance to understand whether these factors could be potential drivers of CBM genesis. In this study, the interspecific relationships and stability of microbial communities in different coal ranks and their relationship to methanogenic potential were evaluated by reanalyzing microbial data in different coal samples that had been obtained.<sup>5</sup> This study aims to (1) describe the co-occurrence network of microbial communities and keystone taxa in coals with different coal ranks and (2) explore factors affecting methanogenic potential.

## 2. MATERIALS AND METHODS

**2.1. Data Sets and Bioinformatics Reanalysis.** In this study, samples of different coal ranks including anthracite ( $n = 9$ ), medium-volatile bituminous ( $n = 9$ ), and high-volatile

bituminous ( $n = 9$ ) were selected, and the main microbial compositions and chemical properties of coal samples were reported.<sup>5</sup> The microbial data were reanalyzed to understand the microbial community network in coals with different ranks. The detailed sample information is given in Table S1.

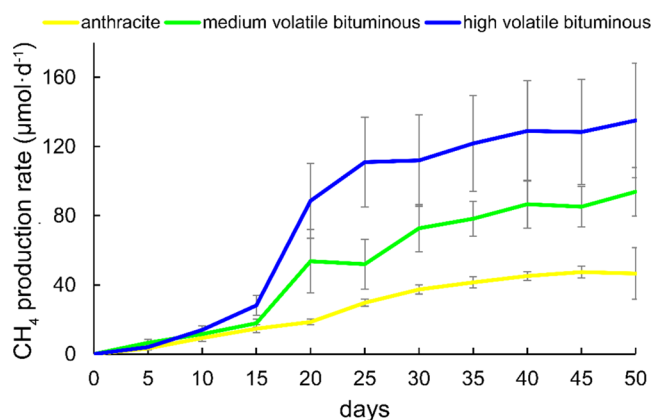
For microbial community (bacteria and archaea) analysis, the reads from 16S genes were merged and the raw sequences were quality filtered using the QIIME pipeline. The chimeric sequences were identified by the “identify\_chimeric\_seqs.py” command and removed with the “filter\_fasta.py” command according to the UCHIME algorithm. The selection and taxonomic assignment of operational taxonomic units (OTUs) were performed based on the SILVA reference data (version 128) at 97% similarity. Reads that did not align to the anticipated region of the reference alignment were removed as chimeras by the UCHIME algorithm. Reads that were classified as “chloroplast”, “mitochondria”, or “unassigned” were removed.

**2.2. Network Construction and Community Stability Analysis.** A co-occurrence network for microbial species was constructed by the SparCC method with a significance of  $P < 0.05$  and a correlation coefficient  $|r| > 0.60$  on the integrated network analysis pipeline (iNAP, <http://mem.rcees.ac.cn:8081/>).<sup>20</sup> For each group, the OTUs detected in above 50% of samples were included for Pearson correlation analysis. The network properties were assessed by the “global network properties and individual nodes’ centralit” module.<sup>20</sup>

The within-module connectivity ( $Z_i$ ) and among-module connectivity ( $P_i$ ) values were calculated by the “module separation and module hubs” module. Based on the  $Z_i$  and  $P_i$  values, the functional genera in co-occurrence work were classified into four topological roles including module hubs ( $Z_i \geq 2.5$ ,  $P_i < 0.62$ ), network hubs ( $Z_i \geq 2.5$ ,  $P_i \geq 0.62$ ), connectors ( $Z_i < 2.5$ ,  $P_i \geq 0.62$ ), and peripherals ( $Z_i < 2.5$ ,  $P_i < 0.62$ ).<sup>21</sup> Among them, module hubs, network hubs, and connectors have been considered the microbial keystone taxa.<sup>22</sup>

After “taxa shuffle” null module-correcting, positive and negative cohesions were calculated based on a connectedness matrix with average positive and negative correlations for each sample, respectively.<sup>23</sup>

**2.3. Analysis of the Methanogenic Potential of Coals.** For each coal sample retaken from  $-80$  °C, 10 g of the sample was cultured in an anaerobic environment using 500 mL sterile culture bottles at 37 °C with 100 mL of minimal salt media that consisted of NaCl (0.5 g/L), MgCl<sub>2</sub> 6H<sub>2</sub>O (0.5 g/L), CaCl<sub>2</sub> 2H<sub>2</sub>O (0.1 g/L), NH<sub>4</sub>Cl (0.3 g/L), KCl (0.5 g/L), KH<sub>2</sub>PO<sub>4</sub> (0.2 g/L), and cysteine hydrochloride (0.5 g/L). All of the sterile culture bottles from the three groups were sealed with butyronitrile plugs, and the headspace air was replaced with high-purity nitrogen. The methane concentration was monitored every 5 days, and the headspace gas was replaced with nitrogen after each monitor to prevent the inhibition of methane production by the methane content. Methane content was determined regularly by gas chromatography with a TDX-01 packed column, and the inlet temperature, column temperature, and detector (TCD) temperature were set at 105, 90, and 120 °C, respectively. The methane content was calculated based on the relative ratio of methane to nitrogen in the headspace air. It found that the methanogenic potential was quite different in coal samples with different ranks (Figure 1).



**Figure 1.** Changes in the methane production rate in coals with different coal ranks. The error bar showed the standard deviation for coals in each coal rank.

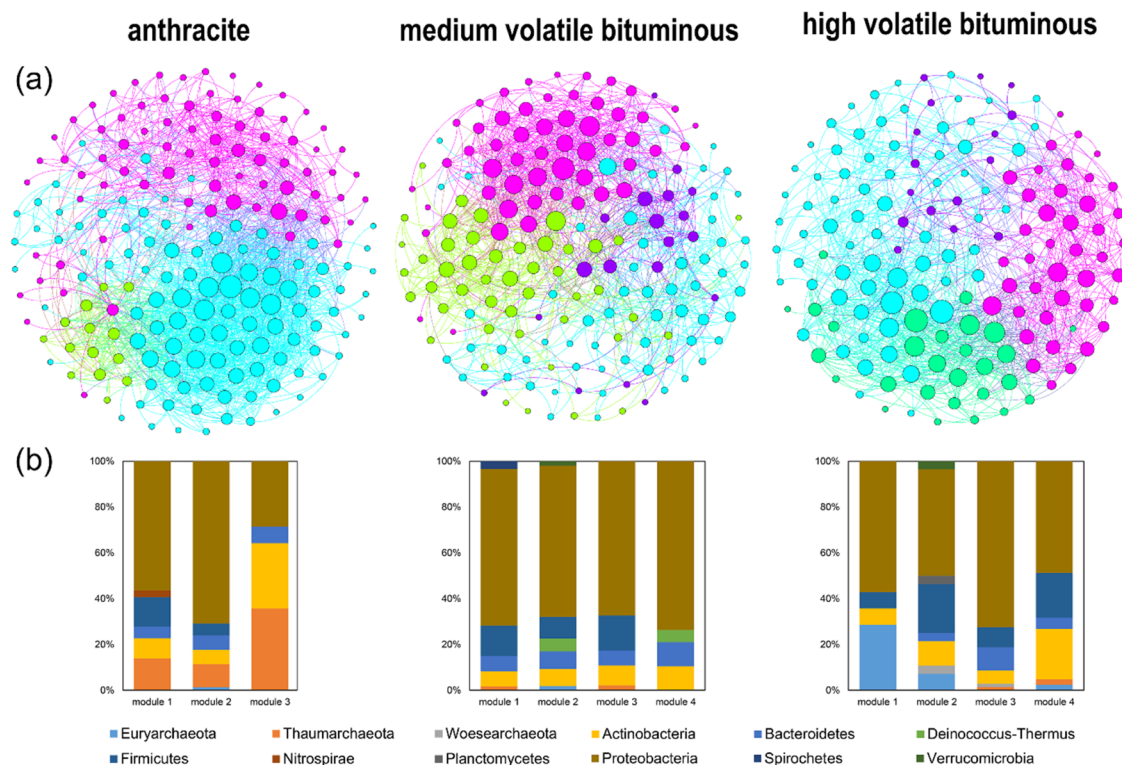
**2.4. Partial Least-Squares Path Model (PLS-PM) Analysis.** To explore the interplay among environmental factors, microbial diversity, keystone abundance, and stability, the framework to evaluate all parameters in networks was built using PLS-PM. PLS-PM was conducted to reveal the effect of keystone abundance on community stability and methanogenic potential by the *plspm* package R v 4.1.2. First, the structural model, which exhibited the relationships between latent variables, was used. Then, the measurement model, which showed the reflective relationships between each latent variable and its block of indicators, was used. After assessing the quality of the outer model, the path coefficient, which represented the

strength and the direction of the relations between variables and predictors, and its significance were calculated for each path. Finally, a pseudo-goodness-of-fit (GoF) measure was used to evaluate the performance of the model.

### 3. RESULTS

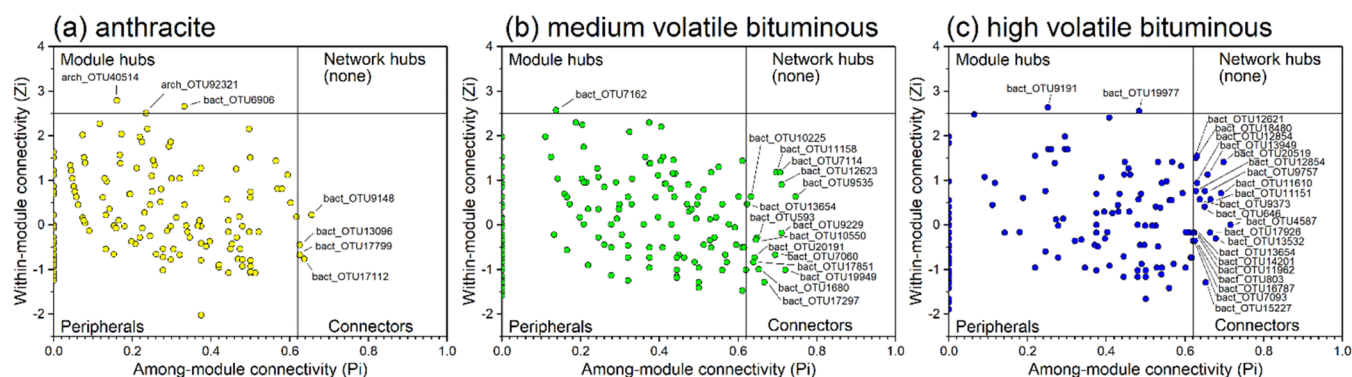
**3.1. Co-occurrence Network among Different Coal Ranks.** Three co-occurrence networks for coals with different ranks were constructed to visualize the microbial interactions (Figure 2A). In the networks, the indices of the average clustering coefficient, average path distance, harmonic geodesic distance, and modularity were larger than those of the corresponding random networks (Table S1), showing the small-world and modular properties. The microbial co-occurrence networks of coals among different ranks are shown with similar topological properties in Table S1. The total nodes ranged from 154 to 195, and the total links ranged from 1146 to 1890 (Table S1). In addition, all of the topological properties of the networks in coals such as the average degree, average clustering coefficient, average path distance, and harmonic geodesic distance did not show great differences in coals among different ranks (Table S1). To summarize, it suggested that the complexity of the microbial co-occurrence network did not change dramatically in coals with different ranks.

**3.2. Modularity and Potential Keystone Taxa.** The overall co-occurrence networks are shown in Figure 2. All three co-occurrence networks could be separated into six modules, respectively (Table S2). However, the large modules with above 10 nodes were concerned, and the number of large modules and the percentages of nodes in large modules ranged



**Figure 2.** Co-occurrence network patterns and relative abundance of microbial communities. (a) Visualization of constructed microbial networks for large modules in coals with different coal ranks based on the OTU level; the modules 1–4 for each network are represented by cyan, magenta, green, and dark violet, respectively. (b) Microbial compositions based on the phylum level in the large modules of the microbial co-occurrence networks in coals with different coal ranks.





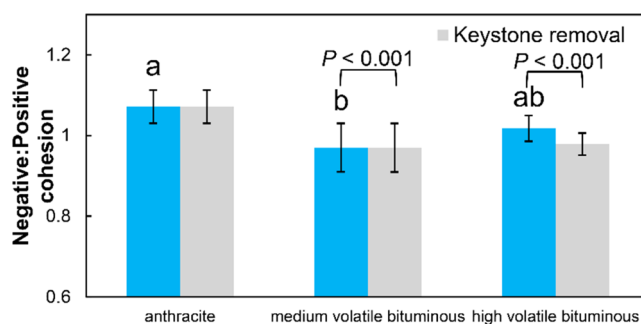
**Figure 3.** Identification of keystone taxa (at the OTU level) among different pH conditions based on their topological roles in networks. (a) Anthracite, (b) medium-volatile bituminous, and (c) high-volatile bituminous. Module hubs are identified as  $Z_i \geq 2.5$ ,  $P_i < 0.62$ , connectors are identified as  $Z_i < 2.5$ ,  $P_i \geq 0.62$ , and network hubs are identified as  $Z_i \geq 2.5$ ,  $P_i \geq 0.62$ .

from 98.05 to 98.88%. The main nodes in large modules are mainly composed of Euryarchaeota, Thaumarchaeota, Woe-searchaeota, Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, Nitrospirae, Planctomycetes, Proteobacteria, Spirochetes, and Verrucomicrobia (Figure 2b). Among them, a great difference was found in the microbial composition in different large modules in coals with different ranks, and these three networks had three, four, and four large modules with seven, nine, and nine phyla, respectively (Figure 2b).

The network of microbial communities with environmental factors showed that the basic properties of coal were associated with different modules in coal microbial networks with different ranks, indicating that the factors affecting different modules of microbial networks in coals with different ranks were quite different (Table S3).

Based on the topological roles for the nodes, a total of 6 module hubs and 40 connectors were identified as the keystone taxa (Figure 3). The number of keystone taxa increased with decreasing coal rank. These keystone taxa were different among different coal ranks, and all of the keystone taxa were identified in large modules. Among them, archaeal keystone taxa were merely detected in the anthracite network and belonged to the Thaumarchaeota phylum. Bacterial keystone taxa mainly belonged to phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. For the level of OTUs (that is, the species level), common keystone taxa were found in different networks. Some of these OTUs belong to the same genus in different networks (Table S4). For example, genus *Herbaspirillum* appeared as keystone microorganisms in all three networks and genera *Corynebacterium 1* and *Pseudomonas* appeared as keystone taxa in bituminous coals.

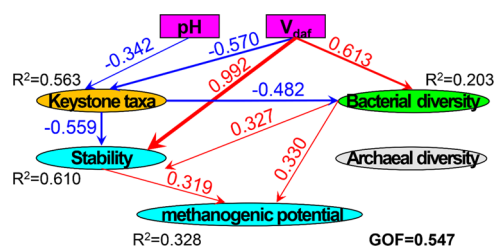
**3.3. Factors for Community Stability and Methanogenic Potential.** The cohesion feature analysis was performed to evaluate the stability of the microbial communities in coals. It showed that there was no difference in the number of positive and negative cohesions in coals with different ranks (Figure S1). The ratio of negative cohesion to positive cohesion denoted by negative:positive cohesion was used to indicate the stability of the microbial community, and a lower value exhibited lower stability of the microbial community in coals (Figure 4). The network in anthracite samples showed the highest negative:positive cohesion value ( $1.07 \pm 0.04$ ), and the network in medium-volatile bituminous showed the lowest negative:positive cohesion value ( $0.97 \pm 0.06$ ). In addition, the



**Figure 4.** Negative:positive cohesions were calculated from OTU abundances between negative:positive cohesions with (blue) and without (gray) keystone taxa. Different lowercase letters above the blue bars indicate significant differences in negative:positive cohesion with keystone taxa among coals with different coal ranks (ANOVA with the Tukey post hoc test,  $P < 0.05$ ). The  $P$  value (paired Student's  $t$ -test) showed the difference between negative:positive cohesions with and without keystone taxa.

removal of key microbial data could reduce the community stability of microbial communities in bituminous coals.

A partial least-squares path model (PLS-PM) was used to comprehensively understand the mechanism of maintaining microbial community stability and controlling methanogenic potential in coals (Figure 5). The GOF of the overall model is 0.547, which was higher than the standard of good fit 0.36 proposed by Wetzels et al.,<sup>24</sup> indicating that the model has a



**Figure 5.** Contribution of factors to microbial community stability and methanogenic potential. Wider arrows indicate greater path coefficients and red and blue lines represent positive and negative correlations, respectively. Insignificant paths are not shown in the model. The keystone taxa, bacterial diversity, archaeal diversity, and stability are denoted by the keystone relative abundance of keystone, bacterial chao1 richness, archaeal chao1 richness, and negative:positive cohesion.  $V_{daf}$  air-dried basis volatile.

high degree of fit. PLS-PM explained 61.0 and 32.8% of the variation in the community stability and methanogenic potential, respectively. The archaeal diversity was not affected by coal properties (mainly, pH and  $V_{\text{daf}}$ ) and was not the core contributor to promoting methanogenic potential. In contrast, bacterial diversity had a positive correlation with  $V_{\text{daf}}$  (path coefficient = 0.613,  $P = 0.024$ ) and a negative correlation with keystone taxa (path coefficient =  $-0.482$ ,  $P = 0.045$ ), which could directly affect community stability (path coefficient =  $0.327$ ,  $P = 0.047$ ) and methanogenic potential (path coefficient =  $0.319$ ,  $P = 0.049$ ). In addition, keystone taxa had negative correlations with pH (path coefficient =  $-0.342$ ,  $P = 0.024$ ) and  $V_{\text{daf}}$  (path coefficient =  $-0.570$ ,  $P < 0.001$ ), and could indirectly affect methanogenic potential by maintaining microbial community stability (path coefficient =  $-0.559$ ,  $P = 0.021$ ). To summarize, these results showed that the keystone taxa played important roles in determining the community diversity, maintaining the microbial community stability, and controlling the methanogenic potential.

#### 4. DISCUSSION

This study considered the co-occurrence network and the community stability in coals with different ranks, which may be related to the biological methanogenic potential of coal seams. However, this information was often ignored in the field of coal microbiology. Since characterizing a microbial community is critical for understanding biogeochemical properties in ecosystems,<sup>25,26</sup> this study reanalyzed the data set from our previous study<sup>5</sup> to construct molecular ecological networks, which was used to provide a new understanding of the relationship between the co-occurrence network and methanogenic potential. In this study, it found that the topological properties of the coal microbial networks with different ranks did not change significantly, indicating the similarity of the coal microbial networks in different ranks. However, the differences in microbial network relationships in coals with different ranks are mainly reflected by various microbial compositions in the large microbial modules. It showed that the microbial communities, which maintain the community stability in coals with different ranks were not specific, and these microbial communities were dominated by bacteria rather than archaea. Besides, the keystone bacteria with a potential network role in the network potentially affected the potential methanogenic potential of coals by regulating community stability and bacterial diversity, but are not directly related to archaeal diversity. In the field of microbial research on coal seams including these referenced research studies, most attention has been paid to the groups related to the formation of biogenic coalbed methane,<sup>27,28</sup> and these studies were a key hub for applying microbial knowledge to practical use. However, coal seams were important habitats for the coexistence of underground microbial communities, and the stable microecology in coal seams was inseparable from the synergy of multiple functional microorganisms. From the perspective of microbial interaction, this study showed that the interspecific relationship among microbes played an important role in restricting the production of biological CBM. As we know, the cooperation of various microorganisms could improve the degradation of coal macromolecular organic matter into substrates that could be utilized by methanogenic archaea, which was a key to the production of biological CBM.<sup>1,6</sup> Therefore, we must consider the interaction of microbial

communities, community stability, and the keystone taxa that maintain community stability in coal seams.

The microbial co-occurrence network could reflect the mutual relationships among microorganisms under different environmental conditions, especially symbiosis, competition, predation, etc. These mutual relationships among microorganisms could directly determine the stability of the microbial community.<sup>29,30</sup> The symbiotic groups in the community can aggregate into modules, and competition or predation among microorganisms can improve the negative cohesion among microorganisms, which is of great significance to the stability and function of the ecosystem.<sup>31,32</sup> Closely related microbes would allow them to occupy favorable ecological niches and communities would become more stable against environmental changes. In this study, all networks exhibited “small-world” characteristics, which were the basis for ensuring microbial stability, and the removal of keystone taxa directly could affect the community cohesion (Figure 4) and further affect the stability of microbial communities.<sup>33</sup>

In general, keystone taxa played a crucial role in microbial community stability due to their unique topological properties, and they were also the core members of linking modules and networks<sup>34,35</sup> and are often considered to have irreplaceable roles in the microbial community structure and function.<sup>36,37</sup> Keystone taxa could be estimated based on the topological roles of nodes in networks, which concatenated microbial communities and provided new insights into the architecture and community stability in microbial co-occurrence networks.<sup>38,39</sup> In this study, bacteria dominated the keystone taxa in the microbial co-occurrence network. The analysis of PLS-PM showed that these keystone taxa could directly regulate community stability. This is similar to the results in other environments. For example, Liu et al.<sup>40</sup> also considered that keystone taxa had the ability to regulate community stability. These taxa were also affected by a variety of environmental factors such as pH.<sup>40,41</sup> Likewise, the keystone taxa were consistently closely associated with microbial network cohesion in coals with different ranks in this study. Once keystone taxa were removed, the stability of each network was significantly reduced, which also indicates the contribution of these keystone taxa to the network stability. In addition, this study found that coals with different ranks or widely different coal seam habitats mainly caused changes in keystone microbial taxa, which might be an important reason for the differences in bacterial community diversity, which was found in previous studies.<sup>5</sup> Therein, the study found that in the coal microbial network of different ranks, no species (OTUs) was the common keystone taxa in different networks, suggesting that these keystone taxa in coals might only work as core hubs in special coal environments. It suggests that specific coal properties or environmental factors are important reasons for microbial taxa to become keystone groups. For example, Liu et al.<sup>40</sup> investigated the microbial network of the Donghu Lake aquatic ecosystem in different seasons. They found that the keystone taxa changed dramatically although the geographical location remained unchanged, and considered that some groups could only become keystone taxa under specific environmental conditions. Similarly, Shade et al.<sup>42</sup> observed microbial community patterns in the eutrophic Lake Mendota ecosystem over many years and also found that some taxa emerged under specific conditions. Interestingly, genera with some specific functions in the microbial network played a role in maintaining microbial stability in different networks

(Table S3). For example, genus *Herbaspirillum* appeared as keystone microorganisms in all three networks, and genera *Corynebacterium 1* and *Pseudomonas* appeared as keystone taxa in bituminous coals. Genus *Herbaspirillum* has attracted wide attention due to its ability to fix nitrogen under microaerobic or anaerobic conditions,<sup>43</sup> besides, it is widely involved in the C–N metabolic process including metabolizing aromatic compounds<sup>44</sup> and reducing nitrate.<sup>45</sup> *Pseudomonas* is a bacterial genus that has been reported to be ubiquitous in coal seam microbial communities.<sup>46</sup> It is precisely because of the presence of multiple functions of this group that it has different metabolic potentials, allowing it to persist and grow in a wide range of coal seam environments and to utilize a variety of carbon compounds under special environmental conditions. Their lifestyle may be opportunistrophic, which is described by Singer et al.<sup>47</sup> Vick et al.<sup>46</sup> observed two *Pseudomonas* species with markedly different metabolic and ecological lifestyles, reflecting the broad metabolic and lifestyle diversity within such taxa, from parasitic to mutually beneficial<sup>48</sup> and free-living lifestyles. Genus *Corynebacterium* has not received much attention in coal seams, but it has been reported that such a genus has the ability to directly synthesize diacetyl from monosaccharides.<sup>49</sup> Such a function could couple diacetyl metabolic pathways and anaerobic respiration, and finally, achieve a redox balance under anaerobic conditions. In summary, these taxa are responsible for the carbon and nitrogen cycle in various environments, which are important for microbial activity in coal environments. However, we know that the identification of keystone groups by such methods is not adequate, and more methods and experimental designs are needed to demonstrate the role of these keystone taxa in the ecological network.<sup>50,51</sup>

In conclusion, this study comprehensively demonstrated the relationship among coal microbial diversity, networks, and methanogenic potential in different coal ranks. Coal ranks did not lead to dramatic changes in microbial community interactions but rather led to changes that affect the keystone microbial composition. These keystone microbes consisted of specific functional groups that contributed to community stability and microbial diversity and ultimately influenced the methanogenic potential of coal seams. Such information has important implications for the geochemical cycle in coals. Together, this study strengthened our knowledge regarding microbial taxa associated with methanogenesis in coal seams.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c02830>.

Detailed sample information of microbial communities in coals, topological properties of the networks in coals with different coal ranks, module EigenGene analysis for correlations between large modules and environmental factors, list of keystone nodes with their taxonomic information, cohesions in coals with different coal ranks, and random forest analyses predicting the importance of keystones to community stability (PDF)

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### Author Contributions

B.L., J.C., and Y.L. conducted the bulk of the data analysis for the study and co-wrote the manuscript. J.C. and Y.L. provided the funding for the study, were involved in the conceptualization of the study, as well as assisted in the writing of the manuscript. All authors read and approved the final manuscript.

### Notes

The authors declare no competing financial interest.

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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