Carbon–Nitrogen–Sulfur-Related Microbial Taxa and Genes Maintained the Stability of Microbial Communities in Coals

Yang Li, Bingjun Liu, *, Jian Chen, and Xuelian Yue

ABSTRACT: Coal microbes are the predominant form of life in the subsurface ecosystem, which play a vital role in biogeochemical cycles. However, the systematic information about carbon–nitrogen–sulfur (C–N–S)-related microbial communities in coal seams is limited. In this study, 16S rRNA gene data from a total of 93 microbial communities in coals were collected for meta-analysis. The results showed that 718 functional genera were related to the C–N–S cycle, wherein N₂ fixation, denitrification, and C degradation groups dominated in relative abundance, Chao1 richness, Shannon diversity, and niche width. Genus Pseudomonas having the most C–N–S-related functions showed the highest relative abundance, and genus Herbaspirillum with a higher abundance participated in C degradation, CH₄ oxidation, N₂ fixation, ammoniation, and denitrification. Such Herbaspirillum was a core genus in the co-occurrence network of microbial prokaryotes and showed higher levels in weight degree, betweenness centrality, and eigenvector centrality. Among them, genera Methanoculleus and Methanoseta showed higher levels in the betweenness centrality index. In addition, the genus Clostridium was linked to the methanogenesis co-occurrence network module. In parallel, the S reduction gene was present in the highest relative abundance of genes, followed by the C degradation and the denitrification genes, and S genes (especially cys genes) were the main genes linked to the co-occurrence network of the C–N–S-related genes. In summary, this study strengthened our knowledge regarding the C–N–S-related coal microbial communities, which is of great significance in understanding the microbial ecology and geochemical cycle of coals.

1. INTRODUCTION
Coal is the most vital fossil fuel on the Earth. The formation of coals is driven by geological events, geologic settings, and microorganisms. Among them, microbes are the predominant form of life in the subsurface ecosystem including coals and play a vital role in biogeochemical cycles, which have accompanied the evolution of coals over tens to hundreds of millions of years.

Microbial activities run throughout the whole process from humus deposition to anthracite formation. During the humus deposition period, anaerobic or facultative anaerobic bacteria participated in the decomposition of peat or low-rank coals in the anaerobic environment; and in the coalification metamorphic stage, microbes in surface water and groundwater infiltrated into coal seams by surface uplifting, which could act on n-alkanes and other organic matters in coals. Previous studies observed that some organic substances in coals could be degraded by a variety of microorganisms following a quasi-step-by-step biodegradation process. The macromolecular substances of coals and/or peats were degraded into single molecules and oligomers by hydrolysis and fermentation bacteria at first, and then some intermediate products were generated by different acidifying bacteria, acetic acid-producing bacteria, and hydrogen-producing bacteria. The resulting products could further generate methane under the action of methanogens. However, these studies mainly monitored the biomarkers in coals, but the important factors influencing the coal biodegradation and the interaction between microorganisms have received extensive attention merely in recent years.

Except for these above C metabolic processes, the biogeochemical processes of N and S also have an impact on the coal biodegradation. Guo et al. detected the microbial taxa related to N metabolism in coal seams, including N₂ fixing taxa and denitrifying taxa. The participation of these microorganisms in N metabolism can increase the N availability of coal seam ecosystems because bioavailable N is a major limiting factor in the extreme oligotrophic environ-

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ments including coal seams. Shi et al. found that microbial N metabolism had an effect on organic matter decomposition in coals such as the decomposition of cellulose and carbohydrate.

In contrast, S in coals is the most notorious environmental pollutant, and its geochemical processes are closely related to the deposition and formation of coals. For example, most of the S in coals was derived from the seawater submerged in the peat swamp during the peat accumulation process. A large amount of seawater sulfate diffused into the bottom peat and was reduced to H2S, S, and polysulfides by microorganisms. In addition, the release of H2S due to sulfate reduction would be detrimental to the methanogenesis process during the coal biodegradation. The process of anaerobic fermentation of coals might also be affected by degraded intermediates and final products (such as sulfides), whose high concentrations affected pH, disrupted cell membranes, prevented protein synthesis, altered hydrogen partial pressure, reduced bioavailability of trace elements, and hindered mass transfer, and thereby disrupted the anaerobic degradation chain. Among these inhibitory compounds, sulfide is formed by the microbial reduction of sulfate and the degradation of S-containing organic matter under anaerobic conditions, and microorganisms involved in the sulfate reduction could compete with other anaerobic bacteria in an environment with low redox potential. On the other hand, the microbial S metabolism process is not necessarily completely detrimental to the biogeochemical processes of coals. Among the bacterial groups, there are a large number of microbial groups closely related to Desulfomonas. These sulfate-reducing bacteria can use kerogen as an end-point autoacceptor or shuttle to oxidize acetic acid or other simple fatty acids, which is the key to the degradation of organic matter in coal seams. Meslé et al. pointed that the depletion of bitumen by solvent extraction resulted in an increase in methane volume in some shales, indicating the methanogenic potential of the shale matrix. These shale-associated microbial communities were able to produce more acetate when grown on the fulvic acid fraction than on ether extracts of the same shale, wherein the microbes were grown under sulfate-reducing conditions rather than under methane-producing conditions.

Figure 1. Relative abundance (a), niche width (b), Chao1 index (c), and Shannon index (d) for the C–N–S-related microbial communities. Difference letters indicated a significant difference at $p < 0.05$. 

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In summary, C−N−S-related microbial communities play an important role in the decomposition of coal organic matter and coal evolution. Therefore, it is of great significance to systematically describe the C−N−S-related microbial communities in coal seams. In this study, 16S rRNA data of microbial composition in coal samples from the NCBI database were extracted and reanalyzed. The study aims to (1) describe the levels of the C−N−S-related microbial communities and functional genes in coal seams, (2) explore the important role of C−N−S-related groups in the microbial community, and (3) clarify the correlation among C−N−S-related groups in coal seams.

2. MATERIALS AND METHODS

2.1. Data Sets. Until February 2022, literature retrieval was conducted through the Web of Science database, and the published papers5,8,21−30 of “coal” and “microbial communities” were retrieved. The fastQ files according to the accession numbers of the 16S rRNA gene data from coal samples from the NCBI database were extracted and reanalyzed. The study aims to (1) describe the levels of the C−N−S-related microbial communities and functional genes in coal seams, (2) explore the important role of C−N−S-related groups in the microbial community, and (3) clarify the correlation among C−N−S-related groups in coal seams.

2.2. Bioinformatics Analysis. 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 reference alignment were removed as chimeras by the UCHIME algorithm. Also, reads that were classified as “chloroplast”, “mitochondria”, or "unassigned" were removed.

The predictive functional abundance was predicted by PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) with "picrust2_pipeline.py" (https://github.com/picrust/picrust2),31 which performed four key steps including sequence placement, hidden-state prediction of genomes, metagenome prediction, and pathway-level predictions. In addition, the additional output file Predicted Enzyme Commission (EC) number copy numbers was used to screen the C−N−S-related microbial genera.

2.3. Data Analysis. To avoid differences in amplified fragments among different samples, microbiological analysis was performed at the genus level according to the classification. The Shannon diversity and Chao1 richness were determined according to the relative abundance of genera. In addition, Bray−Curtis dissimilarity was calculated based on the relative abundance of genera matrix in the Vegan package of R v 4.1.2. Also, nonmetric multidimensional scaling (NMDS) was applied based on the Bray−Curtis dissimilarity by Vegan’s metaMDS function. Spearman’s correlations between the Shannon diversity, NMDS1, and relative abundance of C−N−S-related groups were performed by PerformanceAnalytics package in R. Random forest machine learning was performed with the caret and random forest package in R. These C−N−S-related groups with nonzero abundance values in at least 10% of the samples were preselected and z-score standardized prior to model training. Network analysis was used to explore...
3. RESULTS

3.1. Relative Abundance and Diversity of C–N–S-Related Microbial Communities. Based on the predicted EC number for each OTUs in the coal microbial communities, a total of 718 functional genera related to C (C degradation, methanogenesis, and CH₄ oxidation), N (N₂ fixation, ammoniation, denitrification, and dissimilatory nitrate reduction to ammonium (DNRA)) and S (S reduction) cycles were detected (Table S1). Among the relative abundance of eight microbial groups (Figure 1a), the relative abundance of N₂ fixation taxa was the highest (43.85 ± 2.35%, ranging from 2.40 to 97.08%), followed by denitrification taxa (41.49 ± 2.36%, ranging from 0.68 to 96.04%), and C degradation taxa (32.58 ± 2.25%, ranging from 1.34 to 94.24%). The relative abundance of methanogenesis taxa was the lowest (5.77 ± 1.54%, ranging from 0.00 to 63.98%). In addition, the regularity of the α diversity indexes and niche width was slightly different from that of relative abundance (Figure 1). The Cha01 richness, Shannon diversity, and niche width of denitrification taxa were the highest (71.91 ± 6.74, 2.34 ± 0.12, and 3.84 ± 0.40, respectively).

3.2. Main C–N–S-Related Microbial Genera and Genes. Among 718 functional genera, many microbial communities participated in multiple element cycles (Table S2). For example, the vast majority of methanogens can fix N₂. The top 20 C–N–S-related functional genera are shown in Figure 2. Among them, the genus Pseudomonas has the most C–N–S functions except methanogenesis and showed the
highest relative abundance (12.02 ± 1.97%, ranging from 0.00 to 89.15%). In addition, the genus *Herbaspirillum* participated in C degradation, CH₄ oxidation, N₂ fixation, ammonification, and denitrification, which accounted for 8.84 ± 1.79% (ranging from 0.00 to 86.34%). The methanogen genera such as *Methanobacterium*, *Methanoseta*, *Methanolobus*, *Methanospirillum*, *Methanobrevibacter*, and *Candidatus Methanoperedens* were also the main N₂ fixation groups in coals. In addition, some common anaerobic taxa such as *Clostridium sensu stricto* 1 were also widely involved in the C–N–S cycles (C degradation, N₂ fixation, and S reduction) of the coal seam environment.

Based on the relative abundance of predictive functional genes (Figure 3), the total relative abundance of S reduction genes was the highest (0.21 ± 0.01%), followed by the total relative abundance of C degradation genes (2.46 × 10⁻⁴ ± 0.42 × 10⁻⁴) and denitrification genes (0.82 × 10⁻⁴ ± 0.12 × 10⁻⁴). Among the C-related genes, the *celF* gene (6-phospho-β-glucosidase) and the *pmoA-amoA* gene (methane/ammonia monooxygenase subunit A) had the highest relative abundance of C degradation (1.36 × 10⁻⁴ ± 0.37 × 10⁻⁴) and CH₄ oxidation (5.03 × 10⁻⁶ ± 1.13 × 10⁻⁶), respectively, and the methanogenesis genes showed no difference in the relative abundance. Among the N-related genes, the *nif* genes (*nifH, nifD*, and *nifK*), the *nar/nxr* genes (*narG, narZ, nraA, narH, narY, nrxB, narI, and narV*), and the *nirB* gene had the highest relative abundance of N₂ fixation, denitrification, and DNRA, respectively. Among the S reduction genes, *cysJ*, *cysH*, and *cysD* genes were the highest in the relative abundance (3.41 × 10⁻⁴ ± 0.27 × 10⁻⁴, 3.61 × 10⁻⁴ ± 0.17 × 10⁻⁴, and 3.44 × 10⁻⁴ ± 0.21 × 10⁻⁴, respectively).

3.3. Effect of the C–N–S-Related Group on the Total Microbial Communities. The diagnostic value of microbiome (n = 93) in coals was further evaluated by applying a random forest machine learning classification and regression analysis with the diversity index, relative abundance, and gene abundance of functional genera. The effectiveness of functional genera in reducing uncertainty and variance within the machine learning algorithm was measured by the decrease in mean accuracy for classification and mean-squared error (% Inc. MSE) for regression (Figure 4). The most important diversity indexes of functional taxa for microbial diversity (Shannon diversity and NMDS1) mainly included the diversity (abundance, Shannon diversity, and NMDS1) of denitrification, DNRA, ammonification, N₂ fixation, and C degradation. The most important genes and genera for microbial Shannon diversity mainly included DNRA genes (*nrfA* and *nrfH*), S reduction genes (*dsrB, cysD, cysJ, dsrA, and str*), N₂ fixation genes (*nifH, nifD*, and *nifK*), and *Enhydrobacter*. The most important genes and genera for microbial NMDS1 mainly included denitrification genes (*norC, nosZ, and nirS*), S reduction genes (*cysNC* and *nirS*), and C degradation genes (*MAN2C1, celF, and chitinase*). In addition, the Shannon diversity and NMDS1 of microbial communities were
significantly related to the Shannon diversity of denitrification communities and NMDS1 of DNRA communities, respectively (Figure 4b,d).

In ecosystem studies, a co-occurrence network has become an essential tool for understanding the symbiotic patterns of microbial communities in ecosystem studies. The co-occurrence network of microbial prokaryotes constructed from correlations with \( r \geq |0.60| \) and \( p < 0.05 \) had 161 nodes (including 96 C–N–S-related genera) and 679 edges (Figure 5a). The correlations identified were predominantly positive. The top 20 genera with the highest values of weight degree, betweenness centrality, and eigenvector centrality were listed. Among them, C–N–S-related taxa had 11, 7, and 10 in the top 20 genera of such three node centrality indices (Figure 5b–d).

**3.4. Coupling Relationship between C–N–S-Related Groups.** Pearson correlation showed that the Shannon diversities, NMDS1, and relative abundances of the majority of the C–N–S-related genera (except methanogenesis ones) were significantly related to each other (Figure 6). The methanogenesis group merely had a significantly positive correlation with the N\(_2\) fixation group in Shannon diversity and relative abundance, and methanogenesis genes were also significantly related to N\(_2\) fixation genes at the level of relative abundance.

The network of correlations \( (r \geq 0.60\% \text{ and } p < 0.05) \) between the relative abundance of the C–N–S-related genera included 88 nodes and 154 edges (Figure 7a). All these correlations identified were positive. Multiple modules were shown in the co-occurrence network of the C–N–S-related genera. Two genera *Clostridium sensu stricto* 3 and *Clostridium sensu stricto* 5 linked to the module enriched with various methanogenesis genera. In addition, the genera with higher degree indexes mainly possessed the functions of N\(_2\) fixation, denitrification, DNRA, C degradation, and S reduction except for methanogenesis (Figure 7b).

The network of correlations \( (r \geq 0.60\% \text{ and } p < 0.05) \) between the relative abundance of the C–N–S-related genes...
included 63 nodes and 207 edges (Figure 7c). These correlations identified were predominantly positive. Multiple modules were shown in the co-occurrence network of the C−N−S-related genes and S-related genes (including sir, cysNC, cysH, cysD, cysI, cysN, and cysJ) and celF gene were the main genes linking the co-occurrence network of the C−N−S-related genes.

4. DISCUSSION

This study comprehensively demonstrated the C−N−S-related microbial taxa and functional genes in coals. It was mainly based on the coal microbial data released in NCBI. To avoid OTU sequence differences caused by various amplified primers, the analyzed taxonomic unit was used at the genus level. In the field of coal seam microbial researches including these referenced researches, most attention has been paid to these groups related to the formation of biogenic coal bed methane,33,34 and these studies are the key hubs for applying microbial knowledge to practical production. However, coal seams were important habitats for the coexistence of underground microbial communities, and the stable microbiology in coal seams was inseparable from the synergy of multiple functional microorganisms.

Characterizing the functional properties and diversity is critical for understanding the community assembly and function relationships of biodiversity and ecosystem.35 In particular, the methanogenesis taxa in coal seams, which have attracted wide attention, had the lowest abundance, biodiversity, and niche width among C−N−S-related microbial taxa (Figure 1). It showed that the number and relative abundance of phylotypes36 and the range of environmental conditions that a species may tolerate 37 were lower than those of other functional groups. The C degradation and N2 fixation groups that provided available C and N for coals and the denitrification groups with nitrate as electron acceptor had higher abundance, biodiversity, and niche width (Figure 1). Microbial growth is influenced by many factors, the most important of which is the availability of nutrients. Therefore,
the availability of nutrients determined microbial community assembly.\textsuperscript{38} Although the coal is mainly composed of the C element, the lack of available nutrients (especially available C and N) limits the microbial activity in coal seams.\textsuperscript{39} Therefore, the addition of nutrients\textsuperscript{40,41} was considered to improve the coal bed microbial activities and further stimulate the production potential of biogenic methane. In the coal seam environments where the available nutrients were extremely deficient, the transfer of nutrients and energy along the trophic level during the assimilation and dissimilatory biomass utilization was the basis of the ecosystem.\textsuperscript{42} The C degradation, N\textsubscript{2} fixation, and denitrification could provide available C, N, and energy for the microecology in coal seams and ensure the exchange of metabolites in the microbial communities.\textsuperscript{43}

For functional genes in this study, the total abundance of genes related to the S reduction process was the highest among the different functional genes investigated. The S reduction genes here were mainly dominated by the cys genes but not the dsr genes encoding sulfate respiration (Figure 3). These cys genes were also the core linking the co-occurrence network of the C–N–S-related genes (Figure 7). There are a large number of microorganisms related to S reduction in coal seams. For example, Midgley et al.\textsuperscript{44} found that some fermentative desulfurizing bacteria could produce H\textsubscript{2}S, which might be related to the symphoretic relationship of other bacteria and might also favor coal degradation. Beckmann et al.\textsuperscript{45} considered that a high sulfate concentration and sulfate-reducing bacteria did not prevent the growth of methanogenic archaea, but sulfate-reducing bacteria had limited energy and competed with methanogenic archaea for acetate. This study found that sulfate in coal seams was mainly utilized by bacteria through the assimilatory sulfate reduction pathway. Several cys enzymes were used to synthesize sulfites and convert sulfates into sulfides, and the existence of sulfate utilization enhanced the bacterial ability to produce amino acids, such as cysteine and methionine.\textsuperscript{46} These processes provided biosulfur for the microbial communities in coals. Gene cel\textsubscript{F} was another core gene linking the co-occurrence network of the C–N–S-related genes (Figure 7), which was dominant in C degradation genes (Figure 3). Such a gene was the key gene that encodes glycosidases hydrolyzing O- and S-glycosyl compounds and played a vital role in the degradation of oligo- and polysaccharides in coal degradation.

The study found that there were generally diverse functional microbial groups in many coal seams, among which some microorganisms with a variety of special functions had a high abundance, such as genera \textit{Pseudomonas} and \textit{Herbaspirillum} (Figure 2). The C–N–S functional communities dominated the diversity and composition of microbial communities (Figure 4) and the co-occurrence network of microbial prokaryotes (Figure 5), and the genus \textit{Herbaspirillum} also ranked in the top 20 in all three node centrality indexes. \textit{Pseudomonas} is a bacterial genus that has been reported to be ubiquitous in coal seams.\textsuperscript{47} This is precisely because that such genus has different metabolic potentials, allowing it to persist and grow in a wide range of coal seam environments and to utilize a variety of C compounds under special environmental conditions. Their lifestyle may be opportunotrophic, which was described by Singer et al.\textsuperscript{48} Vick et al.\textsuperscript{49} observed two \textit{Pseudomonas} species with markedly different metabolic and ecological lifestyles, reflecting the broad metabolic and lifestyle diversity within such taxa, from parasitic to mutually beneficial\textsuperscript{50} and free-living lifestyles. Genus \textit{Herbaspirillum} has raised wide attention due to its ability to fix N\textsubscript{2} under microaerobic or anaerobic conditions\textsuperscript{51} in addition, it is widely involved in the C–N metabolic process including aromatic compounds metabolization\textsuperscript{52} and nitrate reduction.\textsuperscript{53} In addition, methanogenesis genera \textit{Methanoculleus} and \textit{Methanosaeta} were the main hub microbes with a higher betweenness centrality index. \textit{Methanoculleus} has been reported in a coal seam in Hokkaido as the dominant methanogen.\textsuperscript{54} Zhang et al.\textsuperscript{55} found that the existence of \textit{Methanosaeta} with \textit{Pseudomonas} could enhance direct interspecies electron transfer and further promote the anaerobic degradation. These methanogens were the terminal carriers for the transformation of coal organic matter into methane and were an important driving force for the geochemical cycle of C, N, S, and other elements between the lithosphere and the atmosphere.

In contrast, the methanogenesis group was merely related to the N\textsubscript{2} fixation group at the genus and gene levels (Figure 6). It fully showed the deficiency of nitrogen source in the coal seams. The N\textsubscript{2} fixation taxa were widely found in many coal
seams, such as Jharia coal bed\textsuperscript{56} and Alberta coal beds.\textsuperscript{57} Here, genera \textit{Clostridium} linked the module that was enriched in various methanogenesis-related genera (Figure 7). These \textit{Clostridium} might exist in a wide pH and temperature range and metabolize a wide range of substrates including cellobiase, glucose, xylase, vanillase, furalacte, lactate, propanol, and formate,\textsuperscript{58} and were considered important substrate suppliers for methanogens.\textsuperscript{59,60} In addition, such taxa contained a variety of regulatory genes responsible for regulating and absorbing N and urea.\textsuperscript{61}

In conclusion, this study comprehensively demonstrated C–N–S-related microbial taxa and functional genes in coals. There are a large number of C–N–S-related groups in coal seams. The inter-relationship of these taxa ultimately affects the microhabitat and has important implications for the decomposition of organic matter and the geochemical cycles in coal seams. Together, this study strengthens our knowledge regarding the microbial diversity and community composition of coals.

\section*{ASSOCIATED CONTENT}

\subsection*{Supporting Information}

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c02126.

Detailed sample information of microbial communities and list of the C–N–S-related genera in coals (XLSX)

\section*{AUTHOR INFORMATION}

Corresponding Author

Bingjun Liu – State Key Laboratory of Mining Response and Disaster Prevention and Control in Deep Coal Mines, Anhui University of Science & Technology, Huainan, Anhui 232001, China; Institute of Energy, Hefei Comprehensive National Science Center, Hefei, Anhui 230031, China; orcid: 0000-0003-4437-0102; Email: lbjandzl@126.com

Authors

Yang Li – State Key Laboratory of Mining Response and Disaster Prevention and Control in Deep Coal Mines, Anhui University of Science & Technology, Huainan, Anhui 232001, China; Institute of Energy, Hefei Comprehensive National Science Center, Hefei, Anhui 230031, China

Jian Chen – Coal Mining National Engineering and Technology Research Institute, Huainan, Anhui 232001, China

Xuelian Yue – Jinning Holding Shanxi Science and Technology Research Institute Co. LTD, Taiyuan, Shanxi 030600, China

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c02126

Author Contributions

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

Notes

The authors declare no competing financial interest.

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

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