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海 外 特 別 研 究 員 最 終 報 告 書

独立行政法人日本学術振興会 理事長　殿

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海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1-1. 用務地（派遣先国名）用務地：　ワシントン大学　（国名：　米　国　）

1-2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
深海底熱水孔コスモポリタン微生物の分布様式の進化的・生態学的形成プロセスの解明

1-3. 派遣期間：令和　4　年　5　月　1　日　～　令和　6　年　4　月　30　日（　731　日間）

1-4.　受入研究機関名及び部局名
受入研究機関名：　ワシントン大学
部局名：　School of Oceanography

Background

Deep-sea hydrothermal vents are one of the most extreme environments where macrofauna thrive, enabled by symbiotic relationships with microorganisms. Members of the class *Campylobacteria* (homotypic synonym of *Epsilonproteobacteria)* represent ubiquitous and dominant mesophilic chemolithoautotrophs in deep-sea hydrothermal environments worldwide. Sulfur-oxidizing species of the genus *Sulfurimonas* have been found dominantly not only in hydrothermal vent environments but also in hydrothermal plumes (Molari et al., 2023), where other sulfur-oxidizing bacteria, such as the SUP05 group of uncultured *Gammaproteobacteria*, have also been dominant (Dick et al., 2013). Although we have identified the distribution patterns of *Sulfurimonas* species in deep-sea hydrothermal environments (Mino et al., 2013), little is known about the phenotypic heterogeneity, interactions with other microbial species, and the forces that generate their heterogeneity. Through the research period, I aimed to acquire the high-throughput cultivation technique for hard-to-cultivate sulfur-oxidizing microorganisms (e.g., members of the SUP05 group) and revealed the possible factors providing speciation among *Campylobacteria*. Because the isolate we obtained during the cultivation experiment could be novel at the family level and possibly has high scientific value in understanding the new functions of *Alphaproteobacteria* as sulfur-oxidizing episymbionts, we also conducted the characterization of this isolate, although this is outside the scope of our original plan.

1. Isolation of sulfur-oxidizers from deep-sea hydrothermal environments and other anoxic environments and genome sequence of SUP05 species

Progress: Two different types of samples from different origins, i.e., deep-sea hydrothermal plume waters and anoxic sediments, were used for isolation experiments. High-throughput cultivation (HTC) experiments were conducted on hydrothermal plume samples obtained in September 2022 and July 2023 from the ASHES hydrothermal vent (depth = 1540 m) located at Axial Seamount, approximately 470 km west offshore Oregon. The filter-sterilized natural seawater media (PS media) or *in situ* deep-sea hydrothermal plume water media was used for the HTC experiments. Filter-sterilized media were amended with thiosulfate and ammonium, and cultivation was performed for up to 14 weeks at 13ºC or 4ºC. We isolated heterotrophic members, including *Pseudomonas*, *Alteromonas*, and *Marinobacter* from these samples. We also conducted HTC experiments in PS media using sludge and sediment samples obtained from Saanich Inlet in 2018. Saanich Inlet, a seasonally anoxic fjord on the coast of Vancouver Island, British Columbia, is known as the environment where members of the SUP05 group represent up to 37% of the total bacterial community (Walsh et al., 2009). Among the isolates, strain G12 isolated from the sediment sample showed more than 97% homology to episymbiont of *Kiwa* sp., a squat crab lobster living in deep-sea hydrothermal vents, as the most similar sequence belonging to *Alphaproteobacteria*, based on BLAST search. The value of the similarity indicates that they belong to the same genus, although they are not the same species. It should be noted that this episymbiont is an uncultivated bacterium, and its role as an episymbiont is not yet understood (Zwirglmaier et al., 2014). Furthermore, the phylogenetic tree, including closely related sequences, showed that G12 forms a clade with sequences of episymbiont of other squat crab lobster species, indicating a different phylogenetic position from the previously isolated *Alphaproteobacteria* (Figure 1). In order to evaluate the genomic diversity and metabolic traits among cultivated SUP05 clade species, we also obtained two more genomes of SUP05 strains isolated from an oxycline in Effingham Inlet, British Columbia (published as Morris and Mino, 2024).

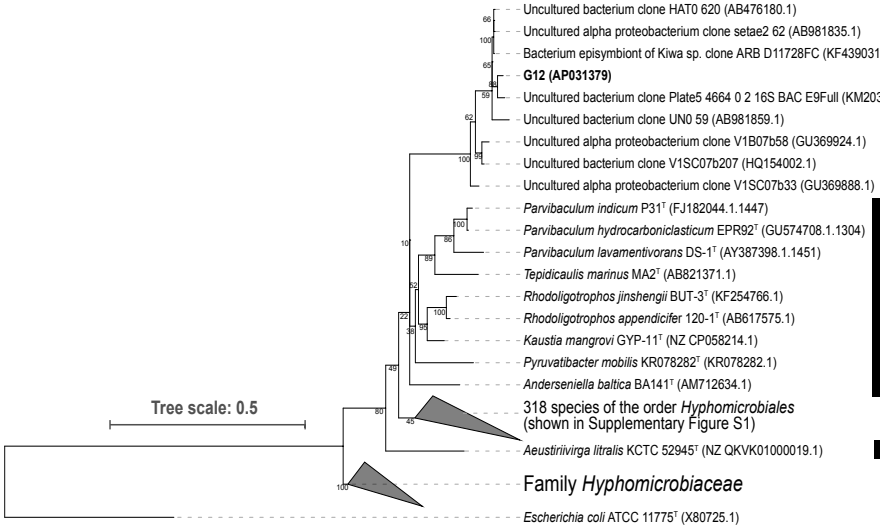

Fig. 1. Phylogenetic position of novel strain G12 based on 16S rRNA gene sequences.

2. Characterization of a novel sulfur-oxidizing *Alphaproteobacteria*

Progress: The novel isolate (G12) was further physiologically and phylogenetically characterized. The Nanopore/Illumina hybrid genome assembly produced a 4,257,309 bp single circular contig with a G+C content of 48.8%. Although the 16S rRNA gene sequence indicated that the strain could be novel at the family level, the taxonomic assignment based on the genome sequence using GTDB-Tk (Li et al., 2019) suggested that the strain is a member of the family *Aestuariivirgaceae* (RED value=0.828). No cultures, metagenome-assembled genomes (MAGs), or single-cell amplified genomes (SAGs) have been reported for this genus and species. A maximum likelihood tree based on 120 conserved protein sequences produced a monophyletic clade with its closest cultured relative, *Aestuariivirga litoralis* (Li et al., 2019). The 16S rRNA gene similarity of G12 against *Aestuariivirga litoralis* was 87.64%. The AAI values of strain G12 compared to *A. litoralis*, *Rhodoligotrophos appendicifer*, and *Rhodoligotrophos defluvi* were 53.1%, 51.6%, and 51.8%, respectively.

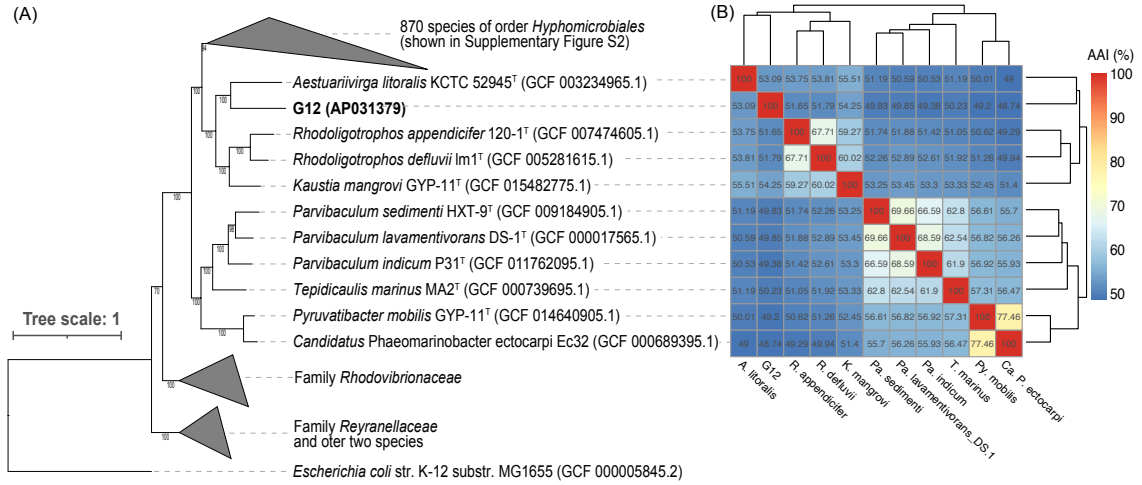

Figure 2. Phylogenomic tree (A) and AAI comparison (B) among closely related species.

We used TFF filter-sterilized seawater media for all growth experiments because attempts to grow strain G12 on artificial media (regular or ½-strength marine broth 2216 and AMP1) were unsuccessful. This is common for cultures we obtain by dilution to extinction on natural filter-sterilized seawater media (Marshall & Morris, 2013). Growth in all experiments was determined by enumerating cells stained by SYBR Green I using a Guava EasyCyte 8HT flow cytometer. Strain G12 showed its growth at a temperature range of 4-23ºC (optimal 17ºC) and a pH range of 4.5–7.8 (optimal 7.8). These characteristics are different from those of the closest species *A. litoralis*, which shows optimum growth at 28 °C and pH 7.0 and no growth in the condition of the presence of NaCl. Anaerobic growth on filter-sterilized seawater media was tested as previously described (Shah et al., 2017), with minor modifications. G12 grew under both aerobic and anaerobic conditions. G12 showed higher growth with thiosulfate under aerobic conditions but not anaerobic conditions (Figure 3). No higher growth was observed under anaerobic conditions with CO_2 in deep-ocean media, which has less dissolved organic carbons than PS media. No motility was observed.

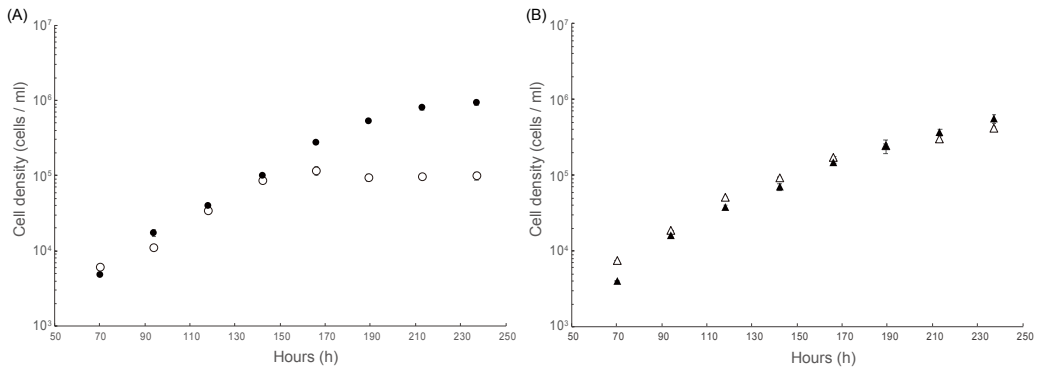

Figure 3. Growth comparison under aerobic (A) and anaerobic (B) conditions. Closed and open symbols indicate media with thiosulfate and without thiosulfate, respectively.

The genome-based metabolic comparison showed that G12 is clustered with similar traits to other closely related heterotrophs; however, the strain also has metabolisms shared with sulfur-oxidizing symbiotic bacterial species that inhibit deep-sea hydrothermal environments, i.e., thiosulfate oxidation, dissimilatory sulfate reduction (Dsr), denitrification, and reductive pentose phosphate cycle (Figure 4). Since strain G12 can use both thiosulfate and organic matter as energy sources, we conducted proteomics to evaluate the proteins associated with sulfur oxidation metabolism and to determine if the metabolism switches during growth phases. A total of 13 L of PS media amended with thiosulfate and ammonium was used for cultivation under aerobic conditions, and 11 L and 2 L were filtered at the log and early stationary phases, respectively. Data-dependent acquisition (DDA) proteomics was performed using a ThermoFisher QExactive with an EASY-nLC 1200 system. Proteins for sulfur oxidation (Sox and Dsr) were detected in both phases. Because the strain lacks *soxCD* genes, strain G12 likely oxidizes thiosulfate to sulfate via incomplete Sox system coupling with the reverse Dsr pathway, contributing to higher efficiency of sulfur oxidation than those relying on the complete *sox* pathway (Klatt and Polerecky, 2015). This combination of sulfur oxidation pathways has also been detected in SUP05 genomes (van Vliet et al., 2021). Abundances of these proteins were not significantly different between the two growth phases, suggesting that strain G12 uses organic matter simultaneously with thiosulfate as energy sources. In addition, the *dsr* genes showed high similarity (~75.4%) to gammaproteobacterial symbionts, suggesting the occurrence of horizontal gene transfers. High similarities to symbiotic bacteria in genes related to autotrophic growth were also observed in other genes, e.g., *rbc* (~90.0%), *napA* (~80.7%), and *nosZ* (~78.7%). Proteins related to nitrate reduction (NapA), nitrous oxide reduction (NosZ), and CO_2 fixation (RbcLS) were also detected at both growth phases. Our results highlight that in addition to well-known sulfur-oxidizing bacterial groups such as *Gammaproteobacteria* and *Campylobacteria*, *Alphaproteobacteria* also have an important role in sulfur, nitrogen, and carbon cycles in deep-sea hydrothermal systems. These results are under preparation for publication.

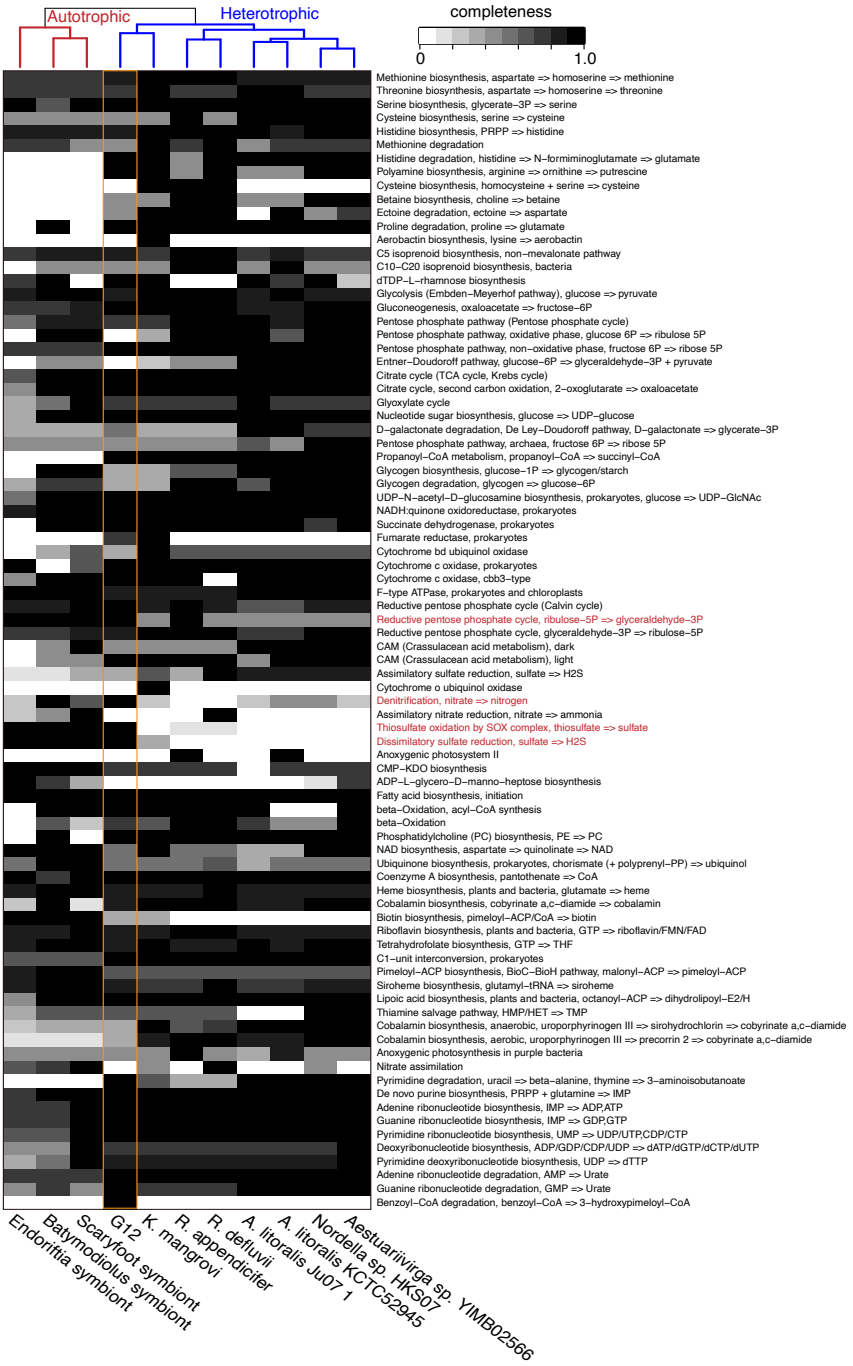

Figure 4. Comparison of metabolic pathways among closely related *Alphaproteobacteria* species and sulfur-oxidizing symbionts in deep-sea hydrothermal environments.

3. Factors for sympatric speciation of sulfur-oxidizing members of *Campylobacteria*

Progress: To understand the possible factors providing sympatric speciation among *Campylobacteria*, strains belonging to the genus *Sulfurimonas* were analyzed for a model case. Closely related strains (>98% similarity in 16S rRNA gene sequences) isolated from the Okinawa Trough that are phylogenetically clustered into two clades by a previous study (Mino et al., 2017, ISME J) were used. A comparison of the complete genomes of a total of 13 species revealed that although the genome sizes and G+C contents differed between the two clades, genome rearrangements were less likely to occur. The reconstruction of metabolic pathways also demonstrated similarities between these clades in key energy metabolisms. Pangenome analysis detected the genes unique to each clade, e.g., genes related to the biosynthesis of O-antigen lipopolysaccharide and to O-glycosylation of flagellin. Interestingly, mutation and recombination rates were different between the two clades, suggesting the main driving force in the sympatric diversification differs among the two clades.

Future works

Detailed physiological and ecological characterization of sulfur-oxidizing *Alphaproteobacteria*, such as CO_2 fixation ability and their distributions, could help to understand their roles in deep-sea hydrothermal ecosystems, including microbe-host species relationships. Modified HTC experiments, such as those targeting anaerobic microbes, possibly capture the diversity of sulfur-oxidizing bacteria, including *Sulfurimonas* and SUP05 group species, and contribute to understanding their interactions in genomes and substrate utilization. Further comprehensive evaluation of genes under selective pressures and their functions may reveal their influence on the diversification of these populations.

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