Candidatus Desulfobulbus rimicarensis, an uncultivated deltaproteobacterial epibionts from the deep-sea hydrothermal vent shrimp Rimicaris exoculata

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ABSTRACT

The deep-sea hydrothermal vent shrimp *Rimicaris exoculata* largely depends on a dense epibiotic chemoautotrophic bacterial community within its enlarged cephalothoracic chamber. Yet our understanding about the shrimp–bacteria interactions is limited. In this report, we focused on the Deltaproteobacterial epibiont of *R. exoculata* from the relatively unexplored South Mid-Atlantic Ridge. A nearly complete genome of a *Deltaproteobacteria* epibiont was binned from the assembled metagenome. A whole-genome phylogenetic analysis reveals that it is affiliated to the genus *Desulfobulbus*, representing a potential novel species, for which the name *Candidatus Desulfobulbus rimicarensis* is proposed. Genomic and transcriptomic analyses reveal that this bacterium utilizes the Wood-Ljungdahl pathway for carbon assimilation and harvests energy via sulfur disproportionation, which are significantly different from other shrimp epibionts. Additionally, this epibiont has putative nitrogen fixation activity, but it is extremely active in directly taking up ammonia and urea from the host or vent environments. Moreover, the epibiont could be distinguished from its free-living relatives by various features, such as lacking chemotaxis and motility traits, a dramatic reduction in biosynthesis genes for capsular and extracellular polysaccharides, enriching genes required for the carbon fixation and sulfur metabolism, and resistance to environmental toxins. Our study highlights the unique role and symbiotic adaptation of *Deltaproteobacteria* in deep-sea hydrothermal vent shrimps.
The shrimp *Rimicaris exoculata* represents the dominant faunal biomass at many deep-sea hydrothermal vent ecosystems along the Mid-Atlantic ridge. This organism harbors dense bacterial epibiont communities in its enlarged cephalothoracic chamber that play an important nutritional role. *Deltaproteobacteria* are ubiquitous in epibiotic communities of *R. exoculata* and their function roles as epibionts are solely based on the presence of functional genes. Here, we describe *Candidatus Desulfobulbus rimicarensis*, an uncultivated deltaproteobacterial epibiont. Compared to campylobacterial and gammaproteobacterial epibionts of *R. exoculata*, this bacterium possessed unique metabolic pathways, such as the Wood-Ljungdahl pathway, as well as sulfur disproportionation and nitrogen fixation pathways. Furthermore, this epibiont can be distinguished from closely related free-living *Desulfobulbus* strains by its reduced genetic content and potential loss of functions, suggesting unique adaptations to the shrimp host. This study is a genomic and transcriptomic analysis of a deltaproteobacterial epibiont and largely expands the understanding of its metabolism and adaptation to the *R. exoculata* host.
INTRODUCTION

The shrimp *Rimicaris exoculata* (1) dominates the macrofauna at many hydrothermal vent sites along the Mid-Atlantic Ridge (MAR), aggregating around active hydrothermal vent chimneys in the mixing zone between electron donor-rich hydrothermal fluids and the surrounding cold oxygenated seawater. Densities of up to 3,000 individuals per m² were observed (2). *R. exoculata* harbors high concentrations of epibiotic bacteria on the inner side of the enlarged cephalothoracic chamber and modified mouthparts, highlighting a symbiosis between the shrimp and its epibionts (3, 4, 5, 6). A number of studies have focused on the nature of this association and its benefits for the shrimp, which have suggested that the shrimp mainly obtains organic matter from epibiotic bacteria that inhabit the cephalothoracic chamber rather than by grazing on free-living bacteria that are associated with chimney walls (5, 7, 8, 9). Furthermore, both inorganic carbon fixation by these chemosynthetic epibionts and transtegumental absorption of dissolved organic matter from epibionts to the shrimp have been demonstrated using isotope-labeling experiments (10).

Recent studies have demonstrated that *R. exoculata* epibiotic communities consist of a high diversity of *Campylobacteria* (previously *Epsilonproteobacteria*), *Gammaproteobacteria*, *Deltaproteobacteria*, *Alphaproteobacteria*, *Zetaproteobacteria*, *Betaproteobacteria* and *Bacteroidetes* (6, 11, 12, 13). Growth of the epibiotic chemolithoautotrophs can be driven by a variety of electron sources, such as reduced sulfur compounds, molecular hydrogen, methane and iron (5, 6, 11, 12, 13). Based on a functional gene survey, two carbon fixation pathways have been highlighted in the epibiotic communities in *R. exoculata* cephalothoracic chambers, namely, the reductive tricarboxylic acid (rTCA) cycle and the Calvin–Benson–Bassham (CBB) cycle (11). A recent metagenomic study performed on a shrimp from the Rainbow hydrothermal vent field revealed that the rTCA and CBB cycles were used for carbon fixation by two filamentous epibionts belonging to the *Campylobacteria* and the *Gammaproteobacteria*, respectively. These epibionts could couple the oxidation of reduced sulfur compounds or molecular hydrogen to oxygen or nitrate reduction (13). In addition, synthetic products from epibiotic chemoautotrophy, such as amino acids, sugars, and vitamins, could be transferred to the shrimp (13).

Meta-omics methods are very useful in adequately identifying and investigating epibiont genetic potential, as most symbiotic bacteria are resistant to *in vitro* cultivation. An early report on
R. exoculata epibionts provided the first insights into potential metabolisms of the epibionts, based on three genomic bins belonging to *Gammaproteobacteria*, *Campylobacteria* and *Zetaproteobacteria* (13). However, these three genome sequences were incomplete and their complete metabolic relationships could not be reconstructed, thereby preventing the interactions with the shrimp host to be predicted. In addition to *Campylobacteria* and *Gammaproteobacteria*, *Deltaproteobacteria* are also frequently detected in epibiotic communities of *R. exoculata* from different deep-sea hydrothermal sites, as revealed by 16S rRNA gene sequencing, fluorescence in situ hybridization (FISH), and metagenomic analysis (11, 12, 13). For example, *Deltaproteobacteria* were highly represented in clone libraries of shrimp epibionts from the Snake Pit hydrothermal vent field (11), and were present in nearly all the life stages of the shrimp at the Logachev vent site (12). These studies tend to indicate that *Deltaproteobacteria* might play a role in shrimp-epibiont interactions. Moreover, Hugler et al. proposed that these epibionts could perform sulfate reduction or sulfur disproportionation only based on the presence of functional genes—the *aprA* gene coding for 5'-adenylylsulfate reductase and the *hynL* gene encoding the large subunit of a [NiFe] hydrogenase (11). Therefore, the ecological functions and potential benefits to the shrimp host remain poorly understood so far, largely due to a lack of genome-level investigations.

In this study, we investigated the *Deltaproteobacteria* associated with cephalothoracic chamber of shrimps sampled from a new hydrothermal vent field named “Deyin,” in the South Mid-Atlantic Ridge (SMAR). Using integrated metagenomics and metatranscriptomics, we assembled and binned the genome of a novel species called *Candidatus* Desulfobulbus rimicarensis, which represents a draft genome of uncultivated deltaproteobacterial epibiont of a deep-sea hydrothermal vent shrimp. Then, we investigated the evolutionary relationships, metabolic activity, and functional dissimilarity of *Candidatus* Desulfobulbus rimicarensis in relation to closely related free-living *Desulfobulbus* strains in order to decipher its adaptation to the shrimp host and to understand the shrimp-epibiont partnership.
RESULTS AND DISCUSSION

Abundance and Localization of the family Desulfobulbaceae. In order to assess the microbial diversity of *R. exoculata* epibionts, nine adult shrimp individuals sampled from the SMAR (Fig. S1) were analyzed by 454 high-throughput pyrosequencing. No obvious differences in microbial community structures were observed among individuals. The epibiotic bacteria mainly consist of Campylobacteria, Gammaproteobacteria, Deltaproteobacteria and Bacteroidetes (Fig. S2) (14). *Deltaproteobacteria* accounted for 0.9 to 4.5% of the epibiotic community of the *R. exoculata* individuals, and *Desulfobulbaceae* accounted for 81.5%-97.9% of the *Deltaproteobacteria* taxa. FISH was also performed to explore the presence of *Desulfobulbaceae* on the cephalothorax sections of the shrimp (Fig. S3). The general probe DSB706 (15), which targets most *Desulfobulbaceae* species, was used, revealing *Desulfobulbaceae* cells at the base of the setae, as previously observed in *R. exoculata* from deep-sea hydrothermal vent sites at the Mid-Atlantic Ridge (11). These *Deltaproteobacteria* were directly attached to the scaphognathite seta, as well as nearby the long filamentous bacteria affiliated to *Campylobacteria* or *Gammaproteobacteria*. This specific localization indicates that *Desulfobulbaceae* are not opportunistic. In addition, *Desulfobulbaceae* species were positively identified by FISH in all tested individuals (*n* = 3). In this study, a total of 16 shrimps were utilized for the various analyses that were performed using 16S rRNA gene amplicon sequencing, FISH, metagenomics, and metatranscriptomics. In all 16 shrimp individuals, *Desulfobulbaceae* bacteria were found as residents of the epibiotic community of the cephalothoracic chamber, indicating that, at the SMAR hydrothermal field, these bacteria were regular epibionts in the *R. exoculata* cephalothoracic chamber.

Genome assembly, characteristics and phylogeny of *Desulfobulbaceae*. *De novo* metagenomic assembly and then binning based on compositional features (tetranucleotide signatures (Fig. S4) and G+C content), followed by alignment, which resulted in several genomic bins. The genomic bin affiliated to *Desulfobulbaceae*, named DR15, was chosen for further analysis. Genome completeness was estimated to be 95.65% and to have only 0.2% contamination based on the checkM method, indicating that the draft genome had a high level of completeness. In order to
determine the taxonomic position of DR15, a maximum-likelihood phylogenetic tree was constructed based on 92 concatenated core genes. The result revealed that strain DR15 was affiliated to the genus *Desulfobulbus*, forming a separate branch with three metagenome-assembled genomes (MAGs) from hydrothermal venting fluids, in the phylogenetic tree (Fig. S5).

The draft genome consisted of 295 contigs (2,921,535 base pairs in length), with an average G+C content of 47.3 mol% (Fig. 1 and Table 1). The genome contained a total of 2,882 protein-coding DNA sequences, resulting in an 83.7% coding density. Approximately two-thirds (1,808) of the protein-coding genes in the genome had the highest BLAST scores against *Deltaproteobacteria* genomes. Of these genes, the majority (81.5%) matched against the family *Desulfobulbaceae*, and 886 coding DNA sequences had top hits with genes of *Desulfobulbus* species. Compared to the genomes of its closest free-living relatives, including *Desulfobulbus mediterraneus* DSM 13871, *Desulfobulbus japonicus* DSM 18378 and *Desulfobulbus propionicus* DSM 2032 (feature summary for these 3 genomes: size, 3.9–5.8 Mb; G+C content, 45.8–58.9 mol%; coding density, 83.4–88.3%), DR15 had the smallest genome size and possessed lower coding density than *D. mediterraneus* DSM 13871 and *D. propionicus* DSM 2032 (Table 1). The genomic size of DR15 was reduced 24–50% compared to the closest related strains. The genome of DR15 had low values of average nucleotide identity (ANI) when compared with genomes of its closest relatives; the highest match was with *D. mediterraneus* DSM 13871 with 66.77% ANI, followed by *D. japonicus* DSM 18378 (66.62%) and *D. propionicus* DSM 2032 (66.53%) (Table 1). These associations are all far below the threshold ANI value of 94–96% for species delineation (16), suggesting that strain DR15 represents a novel species.

Combining the above data, we propose that DR15 should be assigned as a novel species of the genus *Desulfobulbus*, named *Candidatus Desulfobulbus rimicarensis*. Strain DR15 is a deltaproteobacterial representative of the epibionts of this deep-sea hydrothermal vent shrimp.

**Metabolism.** Integrated metagenomic and metatranscriptomic analyses were used to decipher the metabolic potential and transcriptional activity (RNA expression) of *Candidatus Desulfobulbus rimicarensis*. Once onboard, live shrimps were immediately frozen in liquid nitrogen. Although we cannot exclude that the expression of the messenger RNAs may have been partially modified during the sample ascent, we have nevertheless an approximation of the expression *in situ*.
Carbon fixation and central carbon metabolism

In contrast to the epibiotic chemolithoautotrophs previously described in *R. exoculata*, strain DR15 probably uses the Wood-Ljungdahl (WL) pathway for carbon fixation. The epibiont genome contains nearly the complete set of genes required for WL pathway, including homologs of formate dehydrogenase (*fdhA, fdhD, fdhF*), formyl-tetrahydrofolate (THF) synthetase (*fhs*), methylene-THF dehydrogenase (*folD*), methylene-THF reductase (*metF*), methyltransferase (*acsE*), bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase (*acsABC*), phosphotransacetylase (*pta*), and acetate kinase (Fig. S6 and Table S1). The *fchA* gene encoding the formyl-THF cyclohydrolase, which is responsible for converting formyl-THF into methyl-THF, was absent in the draft genome. Previous studies suggest that this gene is not essential for the WL pathway (17, 18). The *metV* gene was also absent; instead, two copies of *metF* gene were found in strain DR15, which could possibly replace the role of MetV in catalyzing the methylenetetrahydrofolate reductase reaction. This agrees with previous studies in *Acetohalobium arabaticum* (18), *Thermus thermophilus* (19), and *Escherichia coli* (20). In addition, the epibiont genome contained genes encoding for THF biosynthesis, corrinoid iron-sulfur protein, and molybdopterin cofactor, which play key roles in single-carbon transfer for synthesizing acetyl-CoA from carbon dioxide and molecular hydrogen (21). This suggests that strain DR15 could synthesize these cofactors to meet the requirements of the WL pathway. In addition, the strain DR15 genome possessed nearly all of the genes needed to reconstruct the complete central pathways, such as the TCA cycle, as well as the Embden–Meyerhof–Parnas, pentose phosphate, gluconeogenesis, and methylmalonyl-CoA pathways (Fig. 3 and Table S1).

All genes required for carbon fixation and central carbon metabolisms described above were found to be actively transcribed in strain DR15 among the studied samples (Table S1). The genes *acsE, fhs, and fdhF* for the WL pathway, *gltA* for citrate synthase in TCA cycle, *talA* encoding transaldolase-associated with the pentose phosphate pathway, *actP* for acetate transport, and *porA* for conversion of acetyl-CoA to pyruvate had the highest transcript abundances (Fig. 3 and Table S1). These data indicated that carbon fixation, acetate uptake, the TCA cycle, and pentose phosphate pathway were active in strain DR15, and around 98-99% of the acetyl-CoA synthesized via WL pathway could be converted into pyruvate, which links the autotrophic WL pathway to heterotrophic metabolism. In addition, a carbonic anhydrase-encoding gene, functioning as a carbon dioxide-
concentrator that elevates inorganic carbon levels for fixation, was highly expressed. These results suggest that this epibiont could be an active chemosynthetic autotroph growing by using the WL pathway for carbon fixation.

The WL pathway was the only carbon fixation pathway discovered in this bacterium. Previous studies have revealed two other carbon fixation pathways, rTCA cycle and CBB cycle, from the epibiotic chemolithoautotrophs of the same vent shrimp species, collected further north of the Mid-Atlantic ridge (13) in epibionts belonging to *Campylobacteria* and *Gammaproteobacteria*. We identified a bacterial symbiont from a vent animal host that is likely to use the WL pathway for carbon fixation. We also report a carbon fixation pathway in a member of the genus *Desulfobulbus*. The pathway has been highlighted in the sulfur-disproportionating bacterium *Desulfocapsa sulfoexigens* (22), which is closely related to strain DR15. In addition, most of the enzymes in the WL pathway encoded in the genome of strain DR15 were most closely related to members of *Deltaproteobacteria* (Fig. S7 and S8). We propose that, as a primary producer in the epibiotic community, the WL pathway could compensate for the rTCA and CBB pathways and could support the growth of the dominant vent fauna.

**Disproportionation of inorganic sulfur compounds**

The biological disproportionation of inorganic sulfur compounds is a microbiologically catalyzed chemolithotrophic process, in which sulfur compounds—such as elemental sulfur, thiosulfate and sulfite—serve as both electron donors and acceptors in order to generate hydrogen sulfide and sulfate. The microbes involved in this type of “inorganic fermentation” or “mineral fermentation” are phylogenetically related to several phyla: *Thermodesulfobacteria* (23, 24), *Firmicutes* (25), *Gammaproteobacteria* (26), and *Deltaproteobacteria* (27, 28, 29, 30, 31, 32). The latter is generally regarded as a lineage of sulfate-reducers (29). Moreover, the capacity to disproportionate inorganic sulfur compounds is relatively common among sulfate-reducers (29). In this study, we have a hypothesis that *Candidatus Desulfobulbus rimiarensis* grew via disproportionation of reduced sulfur compounds, such as thiosulfate, sulfite and elemental sulfur (Fig. 3 and Table S1).

Two thiosulfate reductases (encoding by *phsAB*) were found in the strain DR15 genome and could catalyze the initial step of thiosulfate disproportionation, probably by converting thiosulfate into sulfite and hydrogen sulfide (or less likely to sulfite and element sulfur) (33, 34). Thereafter,
there are two parallel ways for the oxidation of sulfite to sulfate reported in the literature: 1) the sulfate reduction pathway in the reverse direction and 2) the activity of sulfite oxidoreductase (29, 33). The strain DR15 genome contains the complete pathway for dissimilatory sulfate reduction, including ATP sulfurylase (encoded by the gene *sat*), APS reductase (gene *aprAB*), and dissimilatory sulfite reductase (gene *dsrABCD*). Also, genes encoding the APS reductase-associated electron transfer complex (QmoABC) and dissimilatory sulfite reductase-associated electron transport proteins (DsrMKJOP) are present in this genome (Fig. 2B). However, there are no genes that code for sulfite oxidoreductase (Table S1), indicating that strain DR15 likely uses the reverse sulfate reduction pathway to oxidize sulfite to sulfate during thiosulfate disproportionation. The disproportionation of elemental sulfur can also occur via this route, although the first step differs from thiosulfate and is not well described. The capacity to couple growth to the disproportionation of thiosulfate or elemental sulfur has been observed in *Desulfobulbus propionicus* (29, 35), a close relative to the epibiont within the *Desulfobulbaceae* family (Fig. S5). In addition, the predominance of *Desulfobulbaceae* members has also been demonstrated in elemental sulfur-disproportionating enrichment cultures (29). Thus, we propose that strain DR15 may be capable of inorganic sulfur compound disproportionation. Furthermore, transcriptomic analysis revealed that all of the genes involved in the disproportionation of reduced sulfur compounds were expressed (Fig. 2A and Table S1). The genes *aprAB* for APS reductase and *sat* encoding ATP sulfurylase had the highest abundances among all transcripts, followed by *phsAB* for thiosulfate reductase and *dsrABCD* for dissimilatory sulfite reductase. These data, including the expression of *phsAB* genes that are not expressed during sulfate-reduction, confirmed that the disproportionation of inorganic sulfur compounds was active. Therefore, it is likely that thiosulfate disproportionation provides energy for the growth of strain DR15. However, it is also possible that the epibiont might grow via sulfate-reduction under certain conditions.

Previously, the growth of the epibiotic chemolithoautotrophs associated with *R. exoculata* and affiliated to *Gammaproteobacteria*, *Campylobacteria*, *Alphaproteobacteria*, and *Zetaproteobacteria*, was found to be fueled by the oxidation of reduced sulfur compounds, molecular hydrogen, methane, and iron (5, 6, 11, 12, 13). This study demonstrated that chemoautotrophic epibionts of *R. exoculata* are likely to be powered by the disproportionation of inorganic sulfur compounds. Hugler *et al.* had previously made this assumption based on the
detection of aprA sequences from Deltaproteobacteria during a molecular screening of functional genes (11). In the cephalothoracic chamber, energy production through sulfur compounds disproportionation would prevent competition with co-occurring epibionts for energy sources.

Hydrogen oxidation

Genomic analysis revealed that the DR15 genome encoded for four [NiFe]-hydrogenases: two periplasmic hydrogenases group 1 (Hya and Hyb), one cytoplasmic, methyl-viologen-reducing hydrogenase (Mvh), and one membrane-associated energy-converting [NiFe] hydrogenase (Ech) (Fig. S9 and Table S1), while no [FeFe]-hydrogenase genes were detected in the draft genome. [NiFe]-hydrogenases Group 1 is a membrane-bound respiratory hydrogenase, performing hydrogen oxidation linked to quinone reduction (36). Mvh hydrogenases are usually associated with heterodisulfide reductases (Hdr) as large complexes (MvhADG/HdrABC), which are proposed to couple the endergonic reduction of ferredoxin with molecular hydrogen to the exergonic reduction of the heterodisulfide with molecular hydrogen by electron bifurcation (37, 38). In addition, mvhADG genes are sometimes physically located next to hdr genes in some sulfate-reducing organisms and can act as electron acceptors in a process that may involve in electron bifurcation (39). In the DR15 genome, mvhADG genes were also adjacent to the hdr genes (Fig. S6 and Table S1), indicating that the Mvh hydrogenase may perform the same function as in sulfate-reducing bacteria. Ech complexes are widespread in both anaerobic and facultative anaerobic bacteria/archaea and couple the exergonic electron transfer from reduced ferredoxin to H⁺ or the reduction of ferredoxin with molecular hydrogen (21). In the epibiont genome, the gene cluster encoding for Ech complex was present in the same synten than a gene coding for a putative formate dehydrogenase (Table S1). Therefore, the Ech complex is a possible candidate for energy-coupling in the WL pathway of strain DR15 (Fig. S6). This is similar to Moorella thermoacetica, in which Ech activity is coupled to the generation of a transmembrane electrochemical H⁺ gradient (21).

Transcriptomic analysis revealed that all of these hydrogenase genes were expressed. They were involved either in the oxidation of H₂ coupled to the reduction of sulfate, or in electron transfer and cofactor regeneration. We observed that, at the time of sampling, genes encoding hydrogenases were expressed at significantly lower levels than genes involved in disproportionation of inorganic sulfur compounds (Table S1). Hence, based on the transcriptomic data, at the time of sampling the
strain might harvest more energy from sulfur compounds disproportionation than from hydrogen oxidation.

Nitrogen metabolism

Based on the genomic data, DR15 has the potential to use ammonia, urea and molecular nitrogen as nitrogen sources, which represents a wider range of nitrogen sources than previously described for campylobacterial and gammaproteobacterial epibionts of *R. exoculata* (13). The draft genome contains ammonium permeases (Amt), glutamine synthase (GlnA), and glutamate synthase (GltBD) for ammonia assimilation (Fig. 3 and Table S1). The *glnK* gene encoding a regulatory protein P-II was linked to the *amt* gene for ammonia transport, indicating that the nitrogen metabolism in DR15 could be regulated similarly to *E. coli* (40). In addition, the DR15 genome encodes a urea ABC transporter for urea uptake, as well as a urease operon that is involved in urea hydrolysis, suggesting that DR15 could also utilize urea to generate ammonia, which has not been observed in the campylobacterial and gammaproteobacterial epibionts (13). Surprisingly, DR15 was found to potentially be capable of nitrogen fixation. Nearly all of the genes involved in this process were present within the draft genome, including *nifHDK* encoding for a molybdenum-iron nitrogenase, *nifENB, nifU* and *nifS* for assembly proteins, and *nifA* and *ntrXY* for regulator proteins (Table S1).

Similarly, in the sulfur-disproportionating deltaproteobacterium *Desulfocapsa sulfexigens*, all of the genes necessary for nitrogen fixation were observed in the genome (22). Therefore, it is possible that DR15 can grow by utilizing free nitrogen gas as the sole nitrogen source. Symbiotic nitrogen-fixers are known to be associated with wood-boring bivalves, coral, sponges and sea urchins (41). Recently, Petersen et al. provided the first report of nitrogen fixation by a chemosynthetic symbiont in a shallow water bivalve (42). Nitrogen fixation may be more important in the deep-sea environment, especially as nitrogen sources are scarce. However, prior to this study, nitrogen fixation pathways have not been detected in vent animal symbionts. This study reports nitrogen fixation in a chemosynthetic epibiont of *R. exoculata*. In addition, the presence of one denitrification system, including the periplasmic dissimilatory nitrate reductase (Nap) and the nitrite reductase (Nir), indicated that DR15 might have the potential to reduce nitrate to nitrous oxide by dissimilatory nitrate reduction. This ability was also discovered in a gammaproteobacterial epibiont of *R. exoculata* (13).
Transcriptomic data revealed that almost all of the genes required for nitrogen metabolism that were described above were also expressed in strain DR15 (Fig. 3 and Table S1). Among these, the genes involved in ammonia assimilation, such as *amt*, *glnA*, *gltBD*, and *glnK* showed the highest levels of expression, followed by the *urtA* gene for a urea ABC transporter (Fig. 3). In contrast, the genes involved in nitrogen fixation were expressed at relatively low levels. These results indicated that, at the time of sampling, DR15 might utilize ammonia as a main nitrogen source, followed by urea. Moreover, ammonia was assimilated mainly by glutamine synthase (Fig. 3). Nitrogen fixation may be active when the environment is depleted in nitrogen sources, such as ammonia and urea.

**Oxidative stress**

The DR15 genome encodes multiple copies of genes involved in defense against oxidative stress, such as the ruberythrin (Rbr)-rubredoxin (Rbo) oxidoreductase system (Table S1). The system consists of Rbr and Rbo, which have been proposed in *Desulfovibrio vulgaris* as an oxidative stress protection system that is an alternative to superoxide dismutase (SOD) (43). In addition, the genome also encodes a bd-type cytochrome terminal oxidase (Fig. 3 and Table S1). This enzyme reduces molecular oxygen using electrons from the quinone pool in *Desulfovibrio* species (44), thereby protecting cells from molecular oxygen. The presence and expression of the Rbr-Rbo system and of its regulator (PerR), as well as bd-type cytochromes, could indicate that *Candidatus Desulfobulbus rimicaricensis* encounters a wide range of redox gradients as the shrimp swims through the vent environment.

**Amino acid and cofactor biosynthesis**

Strain DR15 can synthesize all 20 amino acids, as all the genes essential for amino acid biosynthesis were present in the genome and were expressed (Table S1). DR15 also contains all genes required for the biosynthesis of selenocysteine, an essential catalytic component for the selenium-containing variant of formate dehydrogenase in the WL pathway (18). In addition, this bacterium has the genetic potential to synthesize vitamin B12, B1, and B6, as well as many other cofactors. Vitamin B12 is essential for both the methyl and carboxyl branches of the WL pathway. The draft genome contains an almost complete set of the genes required for synthesizing cobalamin via precorrin-2 (Table S1). The biosynthesis of biotin, heme and siroheme, riboflavin, folate, and tetrahydrofolate,
pantothenate and coenzyme A, NAD and NADP, and a molybdenum cofactor could also be performed in this bacterium, based on the presence of the required genes (Table S1). However, the genes involved in the biosynthesis of menaquinone are incomplete, which suggests that the epibiont might depend on an external supply of this compound or that these genes were not captured due to missing portions of the draft genome. All genes involved in cofactor biosynthesis were expressed, and the genes required for vitamin B6 biosynthesis exhibited the highest expression levels.

**Comparative genomic analyses suggest adaptations to an epibiotic lifestyle.** A comparative whole-genome analysis revealed the likely adaptive features between symbiotic and free-living *Desulfobulbus* species (45, 46, 47). Genes from three *Desulfobulbus* genomes used in the comparison were subjected to a pan genome analysis. Of these, 930 occurred in both the shrimp-associated and free-living genome pools, and 1553 and 537 genes were specific to the shrimp-associated and free-living pool, respectively. As a shrimp epibiont, strain DR15 displayed unique symbiotic features, such as carbon and energy metabolisms (Fig. 4). The prediction of a functional WL pathway for carbon fixation was only present in strain DR15, with an enrichment of CO dehydrogenase/acetyl-CoA synthase (8 genes in epibiotic vs. 1-2 genes in free-living genomes). The presence of a functional WL pathway in this epibiont might guarantee a steady carbon supply to the host and ensure its ecological success. Regarding sulfur metabolism, strain DR15 has genes that could potentially reduce tetrathionate and thiosulfate, with six genes encoding for tetrathionate reductase and four genes encoding for thiosulfate reductase, whereas these genes were almost completely absent in the genomes of free-living species. Moreover, seven genes encoding for the uptake of glutamate and aspartate were present in the epibiont genome, whereas only one gene was found in three free-living strains, suggesting that strain DR15 may have a capability to uptake glutamate or aspartate, possibly from the shrimp host, while free-living strains would not have this ability. In addition, strain DR15 also shows an enrichment in CRISPR-associated protein, including Cas, Csd, Csm, and Cmr family proteins (16 genes in strain DR15 vs. 2-8 genes in genomes of free-living species) (Fig. 4). The genomic signature is commonly reported as diagnostic of a typical sponge symbiotic life-style (48), here it probably hints at a yet unrevealed role of these proteins in shrimp-epibiont interactions.

The strain DR15 genome can also be distinguished from the genomes of free-living strains as
it lacks genes encoding for several features typically reported in free-living strains. There were no
genes coding for flagellum synthesis or chemotaxis proteins in the DR15 genome, whereas around
64–82 of genes coding for these features were present in the genomes of free-living species (Fig. 4),
suggesting a non-motile lifestyle. Genes responsible for the biosynthesis of capsular polysaccharide
(CPS) and extracellular polysaccharide (EPS) were almost completely lost in strain DR15 (Fig. 4).
CPS and EPS are extracellular polysaccharides common in a wide range of microorganisms that
play important roles, such as protection against environmental stresses, biofilm formation, and
resistance to phagocytosis or antibiotic treatments. The absence of CPS and EPS suggests that strain
DR15 is weakly protected from extracellular stresses, which is compensated by being located inside
the cephalothoracic chamber. By contrast, this characteristic is likely to diminish the barrier between
the symbiont and shrimp cells, thus benefiting shrimp-epibiont interactions and nutrient exchange.
Furthermore, there was also a dramatic reduction in genes involved in resistance to antibiotics and
environmental toxins in strain DR15 genome (Fig. 4), such as, multidrug resistance efflux and
cobalt-zinc-cadmium resistance. Resistance to these toxins in open water is important for the
survival of microorganisms; however, strain DR15 could escape these toxins by being sheltered by
its shrimp host. In addition, the type I restriction-modification involved in DNA metabolism, as well
as the type IV protein and nucleoprotein secretion system involved in membrane transporter, were
also dramatically reduced in the epibiont genome.

Syntrophic association. Multiple symbionts have been found to co-occur in both deep-sea and
shallow-water hosts, such as mussels, worms, shrimps, and snails (13, 49, 50). Stable associations
between multiple symbionts within a host are assumed to be beneficial to each other (51, 52).
Although the co-occurrence of sulfur oxidizers and deltaproteobacterial epibionts raises the
possibility of an internal sulfur cycle that would take place within the shrimp cephalothoracic
chamber (11), this hypothesis is solely based on 16S rRNA gene sequencing and functional gene
surveys. This study probably supports the existence of a syntrophic relationship between sulfur-
disproportionating Deltaproteobacteria, and sulfur-oxidizing bacteria, including Campylobacteria
and Gammaproteobacteria, which are associated with R. exoculata (Fig. 5). Considering that
species of Campylobacteria and Gammaproteobacteria are filamentous in shape (6, 11, 12), these
bacterium anchor to the surface of the scaphognathite setae at one end of the cell and use the other
end to scavenge sulfide compounds from the interior of the cephalothoracic chamber, producing partially oxidized inorganic sulfur compounds (POSCs) via sulfide oxidation. In contrast, deltaproteobacterial epibionts settle close to the surface of the scaphognathite setae, as observed by Hugler et al. (11). These bacteria seem to be able to utilize the POSCs that are either 1) produced by *Campylobacteria* and *Gammaproteobacteria* or 2) directly transferred from the surrounding environments for disproportionation. In return, the sulfide derived from disproportionation could come back to the *Campylobacteria* and *Gammaproteobacteria* for reoxidation (Fig. 5). Thus, if this hypothesis was confirmed, these different epibiotic styles would not compete for energy sources, but rather share a mutualistic relationship with each other in an epibiotic sulfur cycle. These bacterial species could be specialized to fit into micro-niches and build a harmonious relationship with their host. Deltaproteobacterial epibionts would tend to reside in the anoxic or oxic-anoxic interfaces on the close surface of the scaphognathite setae, while *Campylobacteria* and *Gammaproteobacteria* thrive in the oxic zone, on the scaphognathite setae of *R. exoculata*. This syntrophic association would be based on the exchange of reduced and oxidized sulfur compounds, as already described for an oligochaete worm (51).

### Description of *Candidatus Desulfobulbus rimicarensis*

*Candidatus Desulfobulbus rimicarensis* (ri.mi’ca’ren’sis. L. fem. adj. rimicarensis, referring to *Rimicaris exoculata*, the host shrimp where the species was found. Desulfobulbus rimicarensis, a *Rimicaris exoculata* epibiont).

**Properties:** Phylogenetic analysis shows that the novel strain belongs to the genus *Desulfobulbus*, but forms a separate cluster with other members of this genus. The cell colonizes the surface of scaphognathite setae in the cephalothoracic chamber of *R. exoculata*, a shrimp inhabiting deep-sea hydrothermal vents from the Atlantic Ocean. The bacterium was likely to grow chemolithoautotrophically by disproportionation of inorganic sulfur compounds, or molecular hydrogen oxidation coupled to sulfate reduction, under reduced conditions. It has the genetic potential to utilize diverse nitrogen sources, including ammonia, urea, and free nitrogen gas.

**Metabolic activity:** In its natural environment, this bacterium is likely to utilize carbon dioxide as the main carbon source. Growth might be supported by the disproportionation of reduced sulfur compounds. Ammonia and urea might be used as nitrogen sources. The metabolic plasticity and
activity of this deltaproteobacterial epibiont of a shrimp is likely to confer an adaptive advantage to
the shrimp in the highly dynamic hydrothermal mixing zone.

CONCLUSION

Although Deltaproteobacteria are ubiquitous in epibiotic communities of R. exoculata in deep-sea
hydrothermal vent environments (11, 12, 13), their ecological function and symbiotic adaptation to
the shrimp host are not clear. In this report, we described a novel bacterium Candidatus Desulfobulbus rimicarensis, which represents a characterized (while still uncultivated)
deltaproteobacterial epibiont of this deep-sea hydrothermal vent shrimp. Compared to other
epibions that inhabit the cephalothoracic chamber of R. exoculata (13), this bacterium possess es
unique metabolic pathways, such as the WL pathway, sulfur disproportionation (potentially also
sulfate reduction), and nitrogen fixation pathways. We hypothesize that this bacterium is involved
in a syntrophic association with the sulfur-oxidizing campylobacterial and gammaproteobacterial
epibions of the cephalothoracic chamber through the exchange of sulfur compounds of differing
redox levels. In addition, the genome of this epibiont could be distinguished from its free-living
counterparts by its reduced genome size, the lack of chemotaxis and motility traits, dramatic
reduction of genes involved in the biosynthesis of CPS and EPS, lack of resistance towards
environmental toxins, and enrichment of genes required for the carbon fixation and sulfur
metabolism. These genetic modifications suggest that Candidatus Desulfobulbus rimicarensis is
adapted to its shrimp host.
MATERIALS AND METHODS

Shrimp collection and nucleic acid extraction. Vent shrimps were collected using a grabber during the DY115-26 oceanographic cruise leg III on the South MAR (Site SMAR-S029-TVG11, 15.17°S, 13.36°W, 2807 m depth) (Supporting Information Fig. S1), with Zongze Shao as the chief scientist. Phylogenetic analysis of the mitochondrial cytochrome oxidase subunit I (COI) genes (14) showed that they were most closely related to species *Rimicaris exoculata*, which was firstly found in the north Mid-Atlantic Ridge (1). Once aboard, *R. exoculata* specimens were immediately stored in liquid nitrogen and frozen at −80°C for DNA and RNA extractions. In the laboratory, four specimens were dissected under sterile conditions and the mouthparts were immediately used to extract DNA and RNA. DNA was extracted using a modification of SDS-based DNA extraction method (53). Samples were mixed with 13.5 ml DNA extraction buffer, vortexed vigorously for 1 minute and incubated in an orbital shaker at 37°C for 30 min. Then 1.5 ml 20% SDS was added and the samples were incubated in a shaking water bath at 65°C for 1 h. After centrifugation at 6,000 g for 20 min, DNA supernatant was precipitated with phenol, chloroform, and isopropanol. RNA was extracted using a TRI REAGENT procedure (54). After each extraction, DNA and RNA were assessed with a NanoDrop system (Thermo NanoDrop™ 2000, Wilmington, Delaware, USA) and the gel electrophoresis to determine concentration and integrity, and then were sent to the Chinese National Human Genome Center in Shanghai for high throughput sequencing.

Assembly, binning and annotation of individual genome. Metagenomic DNA sequencing was performed with an Illumina MiSeq platform (500-bp library) at the National Human Genome Centre of China at Shanghai, China, according to the manufacturer’s manual. This produced a total of 14,553,576 reads with a total length of 8.7 Gbp. All these reads were imported into CLC Genomics Workbench 6.5 (http://www.clcbio.com) and trimmed using a quality score of 0.01 and a minimum length of 50 bp. Subsequently, the trimmed reads were assembled with the following modified parameters: wordsize, 61; bubble size, 200; minimum contig length of 200 bp. This procedure resulted in 1,030,504 contigs (11,026 contigs ≥ 1000 bp) with a total length of 394,265,091 bp. The contig coverage was calculated by mapping the trimmed reads to reference algorithm using the minimum similarity of 95% of the read length. Binning of the draft genomes
was carried out based on tetranucleotide frequency by utilizing Databionics ESOM-map software (55) with the same parameters as described by Dick et al. (56). Final results were manually curated for species assignment of contigs based on their coverage, GC content and blast results against nr database. Contigs with ambiguous taxonomic assignments were discarded for the rest of the analysis.

Purity and completeness of genome bins were then assessed by CheckM v.1.0.7 (57) using the lineage-specific workflow. Open reading frames (ORFs) were identified using Prodigal (version 2.6.3) (58). The conserved single-copy gene (CSCG) of genome bins were identified by searching identified amino acid sequences against a HMM database of 107 universally prokaryotic genes (59) using hmmsearch with default settings.

Gene annotation of the resulting draft genome was performed by Rapid Annotation using Subsystem Technology (RAST) server (60) and the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP). Metabolic reconstruction of DR15 was performed based on a list of functional genes involved in important metabolic pathways (Supporting Information Table S1), each of which was automatically and then manually curated by comparing the predicted protein sequences with those in GenBank databases. In addition, the CRISPR-Cas system of strain DR15 was searched using the CRISPRCasFinder (https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index). The webservice HydDB (https://services.birc.au.dk/hyddb/) was used for the hydrogenase classification (61).

Metatranscriptomic analyses. The extracted RNA was treated with DNase I (Takara, Japan) to remove genomic DNA. Ribosomal RNA (rRNA) was removed from the total RNA using the Ribo-Zero™ Magnetic Kit (Epicentre, USA). A total of 100 ng rRNA-depleted RNA was used for cDNA library preparation. Sequencing libraries were constructed by NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer’s protocol. The cDNA was directly sequenced using the Illumina HiseqTM2500 platform at the National Human Genome Centre of China at Shanghai, China. The obtained raw 2× 100-bp paired-end reads were subjected to quality control using the next-generation sequencing (NGS) FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) with a quality score of 0.01 and a minimum length of 50 bp, resulting a total of 5.38 Gbp clean data. To depict the gene expression profiling for each genomic bin, the dereplicated, trimmed, and paired-end Illumina reads were then mapped to contigs from the
DR15 genome using Bowtie, version 1.1.1 (http://bowtie-bio.sourceforge.net/index.shtml), with parameters specifically chosen for RNA-Seq quantification (-n 2, -e 99999999, -l 25) (62). FPKM (fragments per kilobase of transcript per million fragments mapped) was used to estimate the expression level of each gene using RSEM-1.2.3 (http://deweylab.biostat.wisc.edu/rsem/) with the default parameters.

**Phylogenetic analysis.** In order to elucidate the taxonomic positions of *Candidatus Desulfobulbus rimicarenosis*, the bacterial core gene-based phylogenetic analysis was carried out using the Up-to-date Bacterial Core Gene pipeline (UBCG) (63). The whole genome sequences of reference taxa were obtained from NCBI database. The 92 concatenated gene sequences were extracted, aligned and concatenated within UBCG using default parameters. A maximum-likelihood phylogenetic tree was inferred using RAxML version 8.2.11 (64) with the GTR+CAT model and 100 bootstrap replications.

**Comparative genomics.** Comparative genome analysis was performed using the Bacterial Pan Genome Analysis pipeline (BPGA) (65). Core genes were detected using the USEARCH program (v. 11.0) (66) extracted from the whole genome sequences of the four strains, with a 50% sequence identity cut-off. The pan genome analysis also complied a set of accessory genes present in at least two or more strains, and unique genes only found in a single strain.

**Fluorescence in situ hybridization.** The FISH protocol was modified based on a previous description Petersen et al. (6). Whole scaphognathite tissues were embedded in the Tissue Freezing Medium (Leica, Germany) and 5 μm thick sections cut with a CM 1850 microtome (Leica, Germany). The sections were collected on Adhesion Microscope slides (Citonex, China). Sections were hybridized in a buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS, 35% formamide) containing probes at an end concentration of 5 ng μl⁻¹ for 3 h at 46°C, then washed for 30 min at 48°C with washing buffer (0.08 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS, 5 mm EDTA), then dipped briefly in MQ water and 96 % ethanol, then air dried. To stain all DNA, sections were covered with 10 μl of a 1 μg ml⁻¹ DAPI solution and incubated for 3-10 minutes, then rinsed with MQ water and 96 % ethanol, then air dried. Branchiostegite sections were hybridized using probes
Eub338 (67) and DSB706 (15). Observations and imaging were performed using both a fluorescence microscope (Leica DM6000B, Germany) and a confocal laser-scanning microscope (Leica TCS SP5, Germany).

**Nucleotide sequence accession number.** Metagenomic and Metatranscriptomic data were submitted to the Sequence Read Archive (SRA) database of NCBI under accession numbers SRX4896442 and SRX4896443, respectively. The draft genome of *Candidatus ‘Desulfobulbus rimicarensis’* was deposited at DDBJ/ENA/GenBank under the accession RKSL00000000. The version described in this paper is version RKSL01000000.

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**CONFLICT OF INTEREST**

The author declare no conflict of interest.
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**FIGURE AND TABLE LEGENDS**

**Figure 1.** The 2.92-Mbp genome and transcriptome of *Candidatus Desulfobulbus rimicarensis*. The outermost ring shows the annotations of the 11 most abundant transcripts in the transcriptome. The second ring (histogram) shows the relative abundance of transcripts based on FPKM (fragments per kilobase of transcript per million fragments mapped). The third and fourth rings (green and red) indicate predicted ORFs on the plus and minus strands, respectively. The fifth ring indicates the location of rRNA and tRNA genes. The sixth and innermost ring display the GC content and GC skew, respectively. Key to transcripts annotations: 1. Adenylylsulfate reductase (AprAB); 2. ATP synthase (AtpABCDEFFG); 3. Sulfatase-modifying factor enzyme 1 (YfmG); 4. Sulfate adenylyltransferase (Sat); 5. Porin-hypothetical protein; 6. Heterodisulfide reductase (QmoABC); 7. Permease (Sulfite exporter TauE/SafE); 8. TusA-related sulfurtransferase; 9. Dissimilatory sulfite reductase (DsrABCD); 10. NADH-ubiquinone oxidoreductase (NuoABCDHIJKLM); 11. Thiosulfate reductase (PhsAB). The FRPM of all genes in the draft genome of ‘*Candidatus Desulfobulbus rimicarensis*’ is provided as an SI (Table S1).

**Figure 2.** A The transcripts abundances of encoding key genes involved in the disproportionation of inorganic sulfur compounds in *Candidatus Desulfobulbus rimicarensis*. B Disproportionation of inorganic sulfur compounds and energy conservation in *Candidatus Desulfobulbus rimicarensis*. Transcript abundance is normalized for gene length and total number of reads per dataset (FPKM). Abbreviations: AprAB, adenylylsulfate reductase; Sat, sulfuradenylyltransferase; DsrABCD, reverse-type dissimilatory sulfite reductase; PhsAB, thiosulfate reductase; DsrMKJOP, sulfite reduction-associated complex; QmoABC, putative quinone-interacting membrane-bound oxidoreductase; SUL, sulfate permease; APS, adenylyl sulfate.

**Figure 3.** Metabolic map reconstructed from the draft genome of *Candidatus Desulfobulbus rimicarensis*. Biosynthetic amino acids, and central vitamin and cofactors are indicated in red on a pink background. Gene transcripts highly expressed are emphasized in purple, and genes with moderate transcript abundances are indicated in orange. Abbreviations: G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; PRPP, phosphoribosyl pyrophosphate; PEP, phosphoenolpyruvate; PG, phosphoglycerate; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartate; Gln, glutamine; Glu, glutamate; Gly, glycine; Ile, isoleucine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; His, histidine; Met, methionine; Cys, cysteine; Cah, carbonic anhydrase; PorA, pyruvate-flavodoxin oxidoreductase; PpsA, phosphoenolpyruvate synthase; Tal, transaldolase; Eno, enolase; GapA, glyceraldehyde-3-phosphate dehydrogenase; PycA, pyruvate carboxylase; GltA, citrate synthase; AcnA, aconitate hydratase; Amt, Ammonium transporter; Urt, urea ABC transporter; GlnA, glutamine synthetase; GlnK, NifHD-Nitrogen regulatory protein P-II; Glt, glutamate synthase; ActP, acetate permease; PTS, phosphotransferase systems; Nuo, NADH-ubiquinone oxidoreductase; DmsE, decacheme c-type cytochrome; CydAB, cytochrome d ubiquinol oxidase; SDH, succinate dehydrogenase; Cyt bc1, cytochrome bc1-type ubiquinol oxidase; Cyt bd, bd-type cytochrome oxidase; For genes present in sulfur disproportionation and WL pathway, see Figure 2 and Figure S6.

**Figure 4.** Major differential genes between *Candidatus Desulfobulbus rimicarensis* and closely
related free-living *Desulfobulbus* strains based on the Bacterial Pan Genome Analysis.

**Figure 5.** Hypothetical model of the sulfur cycle in the gill chamber of *Rimicaris exoculata* showing a syntrophic cycling of oxidized and reduced sulfur compounds between sulfur-disproportionating *Deltaproteobacteria* epibionts, and sulfur-oxidizing epibionts, including *Campylobacteria* and *Gammaproteobacteria*. Abbreviations: POSCs, partially oxidized inorganic sulfur compounds; Cyt, cytochrome; Hyd, hydrogenases; Sqr, sulfide-quinone oxidoreductase; Apr, adenylylsulfate reductase; Dsr, dissimilatory sulfite reductase; Nrf, cytochrome c nitrite reductase; Nif, nitrogenase.

**Table 1.** General genomic features of *Candidatus Desulfobulbus rimicarensis* and its closest free-living relatives.
|                     | Candidatus Desulfobulbus rimicarents DR15 | Desulfobulbus mediterraneus DSM 13871 | Desulfobulbus japonicus DSM 18378 | Desulfobulbus propionicus DSM 2032 |
|---------------------|------------------------------------------|---------------------------------------|-----------------------------------|-----------------------------------|
| Completeness        | draft                                    | draft                                 | draft                             | complete                          |
| ANI (%)             | 66.77                                    | 66.62                                 | 66.53                             | 66.53                             |
| GenBank number      | Bioproject PRJNA479708                   | AUCW01000000                          | AUCV01000000                      | CP002364                          |
| Genome size (bp)    | 2,921,535                                | 4,784,586                             | 5,794,886                         | 3,851,869                         |
| GC Content (%)      | 47.3                                     | 57.6                                  | 45.8                              | 58.9                              |
| Number of protein-coding gene | 2882                                   | 3819                                  | 4802                              | 3255                              |
| Coding density (%)  | 83.7                                     | 85.4                                  | 83.4                              | 88.3                              |
| Isolation source    | Hydrothermal vent shrimp                 | Deep-sea sediment                     | Estuarine sediment                | Freshwater mud                    |
Figure 1.
Figure 2.
Figure 3.
Figure 5.