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# Metagenomic Assessment of a Sulfur-Oxidizing Enrichment Culture Derived from Marine Sediment

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**The biological oxidation of reduced sulfur compounds is a critically important process in global sulfur biogeochemistry. In this study, we enriched from marine sediments under denitrifying conditions, chemolithotrophic sulfur oxidizers that could oxidize a variety of reduced sulfur compounds: thiosulfate, tetrathionate, sulfide, and polysulfide. Two major phylotypes of 16S rRNA gene (>99% identity in each phylotype) were detected in this enrichment culture. In order to characterize sulfide oxidation, we sequenced and characterized one fosmid clone (43.6 kb) containing the group I sulfide-quinone reductase (*sqr*) gene. Interestingly, four putative rhodanese genes were found in this clone. Furthermore, comparative alignment with the closest genome of *Thiomicrospira crunogena* XCL2 revealed that three homologous genes were located within the vicinity of the *sqr* gene. Fosmid clones harboring carbon fixation (*cbbL* and *cbbM*) and denitrification (*narG*) genes were screened, and the phylogeny of the functional genes was analyzed. Along with the comparison between the *sqr*-containing fosmid clones and the relevant γ-proteobacteria, our phylogenetic study based on the 16S rRNA gene and carbon fixation genes suggest the prevalence of chemolithotrophic γ-proteobacteria in the denitrifying cultures. The findings of this study imply that a combination of cultivation and metagenomic approaches might provide us with a glimpse into the characteristics of sulfur oxidizers in marine sediments.**

***Keywords*:** sulfur oxidation, metagenome, marine sediment, sulfide-quinone reductase

Reduced sulfur compounds generated from anaerobic sulfate-reducing bacteria occur frequently in marine sediments, (Sørensen *et al.*, 1981; Detmers *et al.*, 2001). Besides chemical oxidation, microorganisms are known to be involved in the oxidation of reduced sulfur compounds to sulfate at oxic-anoxic interfaces (Jørgensen, 1982; Friedrich, 1998; Ruepp *et al.*, 2000; She *et al.*, 2001; Theissen *et al.*, 2003; Cytryn *et al.*, 2005). However, only a few isolated strains from marine sediments have been shown to be involved in sulfur oxidation (Schutz *et al.*, 1997; Finster *et al.*, 1998; Kamimura *et al.*, 2003). It has also been established that microorganisms that cannot be cultivated *in vitro* reside in a variety of environments (Reinartz *et al.*, 1998; Schutz *et al.*, 1999). The predominant proportion of sulfur oxidizers is currently regarded as undiscovered. As a typical example, many sulfur oxidizers are obligatorily symbiotic, and those symbiotic sulfur oxidations have generally been revealed based on molecular approaches (Caro *et al.*, 2007; Harada *et al.*, 2009).

Metagenomics is a relatively new field of genetics, and permits the study of microorganisms that cannot be readily cultured in the laboratory. One of the most common methods for the identification of environmental microorganisms is based on the analysis of 16S rRNA gene sequences amplified via PCR from various environments. However, molecular identifications based on 16S rRNA gene sequence analysis do not necessarily correspond to metabolic functions. Therefore, PCR techniques for various genes relevant to biogeochemical cycles have been developed for use in functional gene studies of microbial communities [e.g. *nirS*, *nirK*, *nosZ*, and *narG* for denitrification (Scala and Kerkhof, 1998; Braker *et al.*, 2000; Kandeler *et al.*, 2006; Smith *et al.*, 2007)]. Owing to the importance of sulfur oxidation in the biogeochemical sulfur cycle, previous attempts have been made to develop molecular tools to assess the diversity of genes for dissimilatory sulfite reductase (*dsrAB*), sulfur oxidizing system (*soxB*), adenosine-5'-phosphate reductase (*aprA*), and sulfide-quinone reductase (*sqr*) (Wodara *et al.*, 1994; Zaghloul *et al.*, 1994; Schutz *et al.*, 1997; Leloup *et al.*, 2006; Harada *et al.*, 2009). In addition to this functional gene analysis, metagenomic libraries containing large-sized genome fragment inserts have been shown to be useful in the identification of novel genes and predictions of the physiology of uncultivated microorganisms. As a result, previous screenings of metagenomic libraries of large genome fragments are described in the literature, shedding light on the novel roles of microbes in marine and terrestrial habitats (Schleper *et al.*, 2005; Hallam *et al.*, 2006).

The hidden diversity of sulfide-oxidizing bacteria in sediment environments was suggested by the results of an analysis of putative *sqr* genes, encoding one of the key

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enzymes involved in sulfide oxidation, amplified from sediment environments (Pham *et al.*, 2008). In an attempt to characterize sulfur oxidizers from marine sediments via a cultivation-dependent approach, we cultivated sulfur oxidizers from marine sediments under denitrifying conditions. Despite our successful enrichment of sulfur oxidizers, no pure isolates were obtained. In this study, we characterized the enrichment culture via both molecular and metagenomic approaches: analysis of fosmid clones harboring functional gene sequences involved in sulfide oxidation (*sqr*), carbon fixation (*cbbL* and *cbbM*), and denitrification (*narG*).

## Materials and Methods

### Enrichment of sulfur-oxidizing microorganisms from marine sediments

A denitrifying enrichment culture grown on thiosulfate was established using sediment samples obtained from the East Sea, Korea, as briefly described previously (Pham *et al.*, 2008). The marine sediment was collected from the site at the East Sea (E128°35′, N38°20′; depth 650 m) during July, 2005, using a core sampler. The properties of the bottom water at the four sampling sites were described as follows: temperature, 0.5-0.6°C; dissolved oxygen, 6.7-6.8 mg/L; salinity, 34% (w/v); chlorophyll, 0.2-0.02 mg/L; nitrate, 1.5-1.6 mg/L; ammonia 0.052-0.067 mg/L; phosphate, 0.02 mg/L. These characteristics indicate that the sampling location is in a typical oligotrophic marine environment. One gram of the sediment was inoculated into 100 ml of an anaerobic artificial marine salt medium, ($KH_2PO_4$ 0.2 g; $NH_4Cl$ 0.2 g; $Mg_2Cl{\cdot}6H_2O$ 0.4 g; KCl 0.5 g; $CaCl_2{\cdot}2H_2O$ 0.1 g; NaCl 20.0 g; 1 ml each of trace element solutions I and II; 1 ml vitamin solution; 5 mM bicarbonate in distilled water 1 L) (Fennell *et al.*, 2004), containing 5 mM thiosulfate as the sole electron donor and 5 mM nitrate as the sole electron acceptor in a 120 ml serum bottle with a butyl rubber stopper. After inoculation, the bottle was purged with $N_2$ gas for 30 minutes and incubated at 23°C under dark conditions. The culture was propagated for 3 months through successive serial dilutions. Prior to each transfer, the turbidity of the culture medium was indicative of thiosulfate oxidation and cell growth.

Sulfur oxidation was evaluated with 1 mM thiosulfate, 1 mM tetrathionate, 1 mM sulfite, 1 mM sulfide, 0.1 mM polysulfide, and 0.1% (w/v) elemental sulfur under denitrifying conditions. The thiosulfate concentrations were measured via the following protocol (Voroteliak *et al.*, 1993): 0.1 ml of 0.25 M KCN, 0.1 ml of 0.2 M $CuSO_4$, and 0.25 ml of ferric nitrate reagent (prepared from 100 g of ferric nitrate made up to 1 L with 0.4 M nitric acid) were added to 1.5 ml of sample with vortex-mixing between additions, and the absorbance at 460 nm was measured after 5 min of incubation with a NanoDrop spectrometer (ND-1000, Thermo-Fisher Scientific, Inc). The sulfide concentration was estimated using an improved method (Cline, 1969): 0.25 ml of 0.05 M zinc acetate and 0.025 ml of diamine reagent (prepared from 4.0 g anhydrous ferric chloride and 1.6 g N,N-dimethyl-*p*-phenylenediamine sulfate with 100 ml of 6 N HCl) were added into a 0.5 ml sample with vortex-mixing between additions; 1 ml of distilled water was added after 20 min of incubation and the absorbance at 670 nm was measured in a following 15-min incubition with a NanoDrop spectrophotometer (NanoDrop Technologies). The assimilation of other sulfur compounds was observed indirectly via nitrate reduction. Nitrate respiration was determined by measuring the concentration of nitrate remaining in the enrichment culture and the concentration of nitrite accumulated during denitrification (Strickland and Parsons, 1968).

### Amplification and analysis of 16S rRNA genes

Bacterial genomic DNA from the culture was extracted using a commercial genomic DNA extraction kit (Solgent, Korea). In order to analyze the microbial composition of this enrichment culture, we amplified the bacterial 16S rRNA gene sequences using universal primers (27F and 1492R) (Table 1) and constructed a clone library (Weisburg *et al.*, 1991; Park *et al.*, 2006). The purified PCR products were sequenced by Solgent Co. Ltd. (Korea). The partial 16S rRNA gene sequences were assembled with SeqMan software (DNAStar), and compared with sequences of closely-related organisms from the

**Table 1**. Primers used for PCR amplification of bacterial 16S rRNA gene sequences, and *sqr*, *cbbL*, *cbbM*, and *narG* sequences

| Primer name | Target/application | Tm | Sequence (5′→3′) | References |
|---|---|---|---|---|
| Bacterial 16S rRNA gene | | | | |
| Bac-27F | Bacterial 16S rRNA/clone library | 50.5 | AGAGTTTGATCMTGGCTCAG | Weisburg *et al.* (1991), Park *et al.* (2008) |
| Bac-1492R | | 52.1 | TACGGYTACCTTGTTACGACTT | |
| Sulfide-quinone reductase | | | | |
| SQR-G1-475F | Group I *sqr*/clone library | 59.8 | TGYTWYCCGGCBTAYGA | Pham *et al.* (2008) |
| SQR-G1-964R | | 52.3 | GTSACCATSSWTTCRATCAT | |
| Ge-140F | Group IV *sqr*/clone library | 51 | TGGATTCRTCRAACATTTGGGT | |
| Ge-840R | | 53.5 | AATWAGCATMGCRAARTCGAACTC | |
| RubisCo | | | | |
| cbbL-595F | *cbbL*/clone library | 55.4 | GACTTCACCAAAGACGACGA | Giri *et al.* (2004) |
| cbbL-1387R | | 49.3 | TCGAACTTGATTTCTTTCCA | |
| cbbM-663F | *cbbM*/clone library | 69.5 | ATCATCAARCCSAARCTSGGCCTGCGTCCC | |
| cbbM-1033R | | 75 | MGAGGTGACSGCRCCGTGRCCRGCMCGRTG | |
| Nitrate reductase | | | | |
| narG-1960F | *narG*/clone library | 51 | TAYGTSGGSCARGARAA | Philippot *et al.* (2002) |
| narG-2650R | | 50.7 | TTYTCRTACCABGTBGC | |
| Fosmid vector | | | | |
| FosmidvectorF | Vector/end sequencing | 67.6 | GGATGTGCTGCAAGGCGATTAAGTTGG | Shizuya *et al.* (1992) |
| FosmidvectorR | | 60.5 | CTCGTATGTTGTGTGGAATTGTGAGC | |

GenBank database in order to determine the approximate phylogenetic affiliations using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). Sequence alignments were conducted, then edited with the Clustal_X (Thompson *et al.*, 1997) and the BioEdit program (Hall, 1999). The evolutionary distances were calculated via the algorithm of the Kimura two-parameter model (Kimura, 1983). The phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) and the MEGA 3 Program (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

### Construction of the metagenomic library

The metagenomic library was constructed using a CopyControl™ Fosmid Library Production kit (Epicentre, USA) in accordance with the manufacturer's protocols. In brief, purified DNA (ca. 500 μg/μl) was enzymatically treated to generate blunt-ended DNA fragments and subsequently ligated into the fosmid vector pCC1FOS (Epicentre, USA). After *in vitro* packaging into lambda phages, transductant fosmids were introduced into *Escherichia coli* EPI300-T1®, and the infected *E. coli* cells were plated on Luria-Bertani (LB) broth containing 12.5 mg/ml of chloramphenicol. The plates were then incubated for 24 h at 37°C prior to colony selection. Single colonies were inoculated onto 13 96-well plates with the same medium, and incubated for 1 day at 37°C.

We screened metagenomic clones containing genes involved in sulfur compound oxidation (*sqr*), carbon fixation (*cbbL*, *cbbM*), and nitrate respiration (*narG*) using PCR amplification of metagenomic library pools from approximately 1,000 fosmid clones, a procedure that was used for screening archaeal *amoA* from soil fosmid clones (Schleper *et al.*, 2005). Primers used for amplification of *sqr, cbbL, cbbM*, and *narG* are described in Table 1 (Elsaied and Naganuma, 2001; Kandeler *et al.*, 2006; Meyer *et al.*, 2007; Pham *et al.*, 2008). The phylogenetic trees of functional genes were constructed and analyzed based on nucleotide sequences as described above.

### Fosmid clone sequencing and ORF (Open Reading Frame) analysis

A fosmid clone 9F08 harboring the group I *sqr* gene was selected and the inserted DNA of the clone was sequenced entirely as described previously (Jeon *et al.*, 2009). DNA manipulations were conducted in accordance with standard procedures (Radford *et al.*, 2001). The clones were purified via alkaline lysis (Birnboim and Doly, 1979) and the mechanically sheared DNA (1.53 kb) of the fosmid was ligated to pBluescript SK for shotgun sequencing. DNA sequencing reactions were conducted using a Big Dye kit (Applied Biosystems) and analyzed with an ABI-3100 automated sequencer (Applied Biosystems). The DNA sequences were assembled using the Vector NTI Advance program (InforMax, USA). Gap filling was conducted via the primer-walking method. Open reading frame (ORF) analysis was conducted using the ORF Finder of NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Gene modeling was performed using the Glimmer (Delcher *et al.*, 1999), and GeneMark.hmm PROKARYOTIC Version 2.5a programs (Lukashin and Borodovsky, 1998).

The functional annotation of protein coding regions was achieved using the BLASTP and PSIBLAST programs. Transmembrane domains in the identified proteins were predicted using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (Krogh *et al.*, 2001). Comparisons were conducted between fosmid clone 9F08 and *Thiomicrospira crunogena* XCL2 using the web-based Artemis Comparison program (Carver *et al.*, 2005).

### Nucleotide sequence accession number

The 16S rRNA and 9F08 fosmid sequences obtained in this study are available under the following EMBL-EBI accession numbers: Fosmid 9F08, GU177851; *narG*, GU198378; 16S rRNA gene, GU198379 and GU198381; *cbbL*, GU198382; *cbbM*, GU198387; *rplS*, GU362413.

## Results and Discussion

### Sulfur oxidation and bacterial community of the enrichment culture

We preliminarily reported the presence of two *sqr* groups among the six classified groups in the sulfur-oxidizing enrichment culture (Pham *et al.*, 2008). The results of the sulfur oxidation test demonstrated that the enrichment culture could utilize sulfide, thiosulfate, tetrathionate, and polysulfide as energy sources. Thus far, our effort to isolate pure cultures from the enrichment consortium has not been successful. For analysis of the microbial community, we randomly selected and sequenced 56 clones from a 16S rRNA gene library and detected two dominant phylotypes (>99% similarity amongst each phylotype's sequences), 16S-6 (54 clones) and 16S-9 (2 clones), belonging to γ- and ε-proteobacteria, respectively (Fig. 1). The 16S-6 phylotype was related to *Thioalkalispira microaerophila* ALEN1 (94% 16S rRNA gene sequence similarity). The strain ALEN1 was enriched and isolated from hypersaline and alkaline lakes using thiosulfate as a sole electron donor under denitrifying conditions (Sorokin *et al.*, 2002). The strain ALEN1 can oxidize thiosulfate, sulfide, polysulfide, and elemental sulfur to sulfate. The 16S rRNA gene sequence of phylotype 16S-9 was closely related to *Thiomicrospira denitrificans* (each clone with 97% similarity) (Sievert *et al.*, 2008). In the 16S rRNA gene clone library analysis, strain 16S-6 was dominant over strain 16S-9.

### Analysis of *sqr*-containing fosmid clones

Sulfide is the most reduced form of sulfur among reduced sulfur compounds, and its oxidation is assumed to be a key step in the biogeochemical sulfur cycle (Friedrich, 1998). In the previous study, we surveyed the diversity of *sqr*, one of the sulfide oxidation genes, in marine sediments (Pham *et al.*, 2008). In this study, we obtained and analyzed the *sqr*-containing genome fragments from the enrichment cultures in order to characterize sulfide oxidation.

Although three fosmid clones containing the group I *sqr* genes related to the γ-proteobacteria were acquired among 1,200 fosmid clones, we were unable to identify *sqr* gene clones related to the ε-proteobacteria, which could be retrieved only via PCR amplification (Pham *et al.*, 2008). The *sqr* sequences from the three clones were identical to each other, and one of the clones (9F08) was selected and shotgun-sequenced.

The metagenomic fragment, designated 9F08, was approximately 43.6 kb in length with a G+C content of 60%, similar to that of other known alkaliphilic sulfur oxidizers, *T. microaerophila* (59%) and *Thioalkalivibrio* (61-66%) (Sorokin *et al.*, 2002). We annotated the functions of 32 ORFs on clone 9F08 (Table 2) using the BLASTP and PSIBLAST programs against the NCBI database. The results demonstrated that the 27 putative proteins were significantly similar to known functional genes. On the other hand, five others were hypothetical or

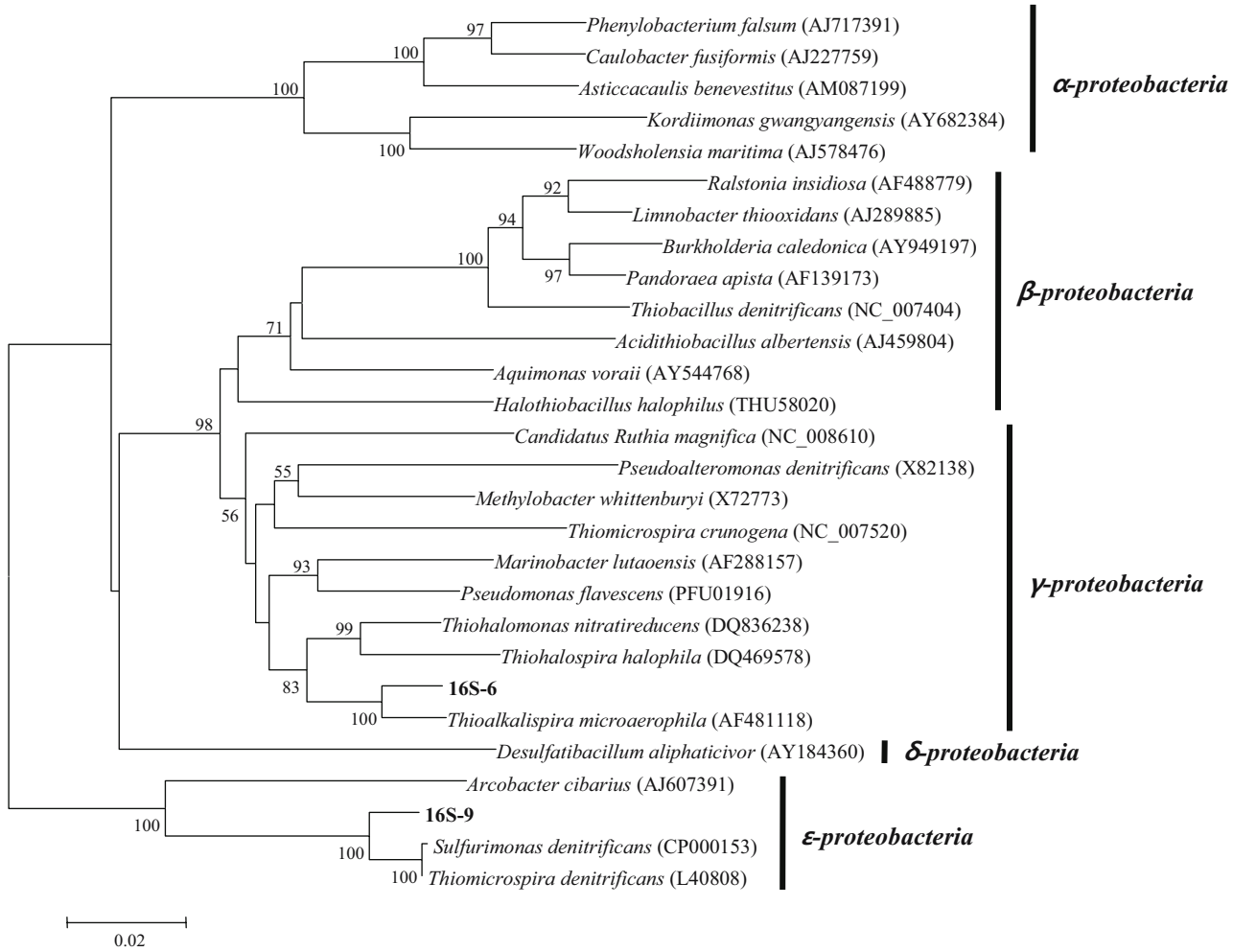


**Fig. 1.** Phylogenetic position of bacterial 16S rRNA gene sequences (about 1,500 bp) amplified from the enrichment culture. Branching patterns supported by more than 50% bootstrap values (1,000 iterations) by means of neighbor-joining were denoted by their respective bootstrap values. Clones obtained in this study are indicated in bold. The reference sequences were selected to indicate the relatives to the sequences determined in this study. The scale bar represents a 2% estimated sequence divergence.

uncharacterized proteins (Table 2). The SQR (ORF 21), consisting of 425 amino acids, showed the highest levels of amino acid sequence similarity (72%) with *T. crunogena* strain XCL2, which was isolated from a deep-sea hydrothermal vent. The strain XCL2 is also capable of using diverse reduced sulfur compounds [sulfide, thiosulfate, elemental sulfur, and sulfide minerals (e.g., pyrite and chalcopyrite)] as electron donors (Scott *et al.*, 2006). When we compared the gene organization of clone 9F08 with that of the genome (NC_007520) of *T. crunogena* strain XCL2, four putative rhodanese-related sulfurtransferase genes (ORF-18, 19, 23, 26) were identified by their sequence homology (Fig. 2). Rhodanese is a ubiquitous protein that catalyzes the transfer of a sulfur atom from thiosulfate (rhodanese) or 3-mercaptopyruvate (mercaptopyruvate sulfurtransferase) to thiophilic sulfur acceptors such as cyanide *in vitro* (Bordo and Bork, 2002) and may play a role in thiosulfate oxidation. Although four rhodanese-like genes are localized around *sqr* in the 9F08 clone, they are dispersed throughout the genome of *T. crunogena* XCL2 (See Fig. 2). Although rhodanese breaks the S-S bond present in thiosulfate, generating sulfur and sulfite (Tabita *et al.*, 1969), an essential involvement in thiosulfate oxidation remains to be shown by biochemical studies. Upstream of *sqr*, ORF-20, which is significantly similar to a transcription factor of *T. crunogena* XCL2 (76.2% amino acid similarity), was found in the same orientation as *sqr*. This gene and *sqr* were separated by 183 nucleotides. Considering that SQR is an inducible protein in cyanobacterial systems and that regulatory proteins are located in the vicinity of *sqr* (Bronstein *et al.*, 2000), this small protein may perform a crucial function in the regulation of *sqr* expression.

Besides *sqr*, the two genomes shared three homologous genes in the vicinity of *sqr*: 1) transcription-repair coupling factor (ORF-22), 2) siroheme synthase (ORF-25), and 3) the lipoprotein releasing system (transmembrane protein and LolC/E family) (ORF-11) (Fig. 2). Among them, ORF-25 is indirectly related to sulfur metabolism, since siroheme has been identified in sulfite reductase, which catalyzes the six-electron reduction of sulfite to sulfide (Murphy *et al.*, 1974). Recently, the *in vivo* role of 'reverse' siroheme sulfite reductase

**Table 2.** Identified genes in fosmid 9F08. The reliability of predicted protein genes was affirmed by comparison between GeneMark.hmm (GMHMM) and Glimmer 3.02 ( Lukashin and Borodovsky, 1998; Delcher *et al.*, 1999).

| ORF | Nucleotide range (protein size, in no. of amino acids) | Dir[a] | Predicted gene | Most similar homolog (e-value)[b] |
|---|---|---|---|---|
| 1 | 847..3702 (952) | - | Predicted signal transduction protein containing a membrane domain, an EAL and a GGDEF domain | NC_007645 HAHCH (4e-135) |
| 2 | 3877..6834 (986) | - | Putative two-component sensor | CP000555 METPE (4e-66) |
| 3 | 7109..7405 (99) | + | Uncharacterized protein | NA |
| 4 | 7388..8329 (314) | - | 3-deoxy-D-manno-octulosonate cytidylyltransferase | CP000647 KPMGH (5e-86) |
| 5 | 8432..11059 (876) | - | Lipid A export ATP-binding/permease protein MsbA | NC_007404 THIODE (2e-166) |
| 6 | 11050..11595 (182) | + | Biopolymer transport protein ExbD/TolR | AE015451 PSEPU (1e-12) |
| 7 | 11520..12131 (204) | + | Probable tolQ-type transport protein | NC_009656 PSEAE (5e-29) |
| 8 | 12197..14494 (766) | - | DNA internalization-related competence protein ComEC/Rec2 | NC_007404 THIODE (5e-95) |
| 9 | 14508..15128 (207) | + | Uncharacterized conserved membrane protein | AE017340 IDILO (3e-49) |
| 10 | 15139..15831 (231) | - | Lipoprotein ABC transporter, ATP-binding protein LolD | NC_002977 METCA (4e-73) |
| 11 | 15824..17071 (416) | - | Lipoprotein ABC transporter, permease protein LolE | NC_002977 METCA (5e-138) |
| 12 | 17455..17832 (126) | - | metal-dependent phosphohydrolase, HD domain protein | CP000462 AERHY (4e-04) |
| 13 | 17860..18255 (132) | - | metal-dependent phosphohydrolase, HD domain protein | CP000462 AERHY (2e-05) |
| 14 | 18610..20976 (789) | - | Putative GTP pyrophosphokinaseRelA | CT573326 PSEEN (9e-141) |
| 15 | 21031..23160 (710) | - | Putative radical S-adenosylmethionine domain protein | CP000438 PSEAE (0.0) |
| 16 | 23309..23572 (88) | - | Hypothetical protein | BA000030 STRAV (2.6) |
| 17 | 23661..24737 (359) | + | Arginase/agmatinase/formiminoglutamate hydrolase, arginase family | CP000361 ARCBU (4e-97) |
| 18 | 25473..26126 (218) | + | Rhodanese-like protein | NC_007404 THIODE (9e-62) |
| 19 | 26162..26905 (248) | + | Rhodanese-like protein | NC_007404 THIODE (5e-60) |
| 20 | 26915..28303 (463) | - | Probable transcriptional regulator | AM406670 AZOAR (1e-86) |
| 21 | 28486..29760 (425) | - | Sulfide-quinone reductase | NC_007520 THIOCR (1e-151) |
| 22 | 30030..33596 (1189) | + | Transcription-repair coupling factor | CP000438 PSEAE (0.0) |
| 23 | 34123..34845 (241) | + | Rhodanese-like protein | NC_007404 THIODE (1e-41) |
| 24 | 34889..35554 (222) | - | Hypothetical protein | NC_007404 THIODE (8e-23) |
| 25 | 36080..37486 (469) | - | Siroheme synthase | CT573326 PSEEN (4e-146) |
| 26 | 37708..38544 (279) | + | Rhodanese-like protein | NC_007645 HAHCH (9e-49) |
| 27 | 38509..39810 (434) | - | Seryl-tRNAsynthetase | NC_002977 METCA (3e-172) |
| 28 | 39843..40154 (104) | - | Conserved hypothetical protein | NC_002977 METCA (2e-20) |
| 29 | 40136..40525 (130) | - | crcB protein domain protein | NC_002977 METCA (1e-29) |
| 30 | 40555..41931 (459) | - | ATPase, AAA family | CP000058 PSESY (5e-159) |
| 31 | 41943..42590 (216) | + | Outer membrane lipoprotein carrier protein LolA | NC_002977 METCA (2e-16) |
| 32 | 42623..43630 (336) | - | Cell division protein FtsK | NC_007645 HAHCH (2e-131) |

NA, Not applicable

[a] direction of transcription, namely, reverse (-), forward (+);

[b] The proteins are designated by their accession numbers followed by the abbreviated species name: *Thiobacillus denitrificans* ATCC 25259 (THIODE); *Pseudomonas putida* KT2440 (PSEPU); *Hahella chejuensis* KCTC 2396 (HAHCH); *Thiomicrospira crunogena* XCL-2 (THIOCR); *Methylococcus capsulatus* str. Bath NC_002977 (Metcalf *et al.*, 2008); *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (AERHY); *Methylibium petroleiphilum* PM1 (METPE); *Pseudomonas aeruginosa* UCBPP-PA14 (PSEAE); *Klebsiella pneumoniae* subsp. *Pneumoniae* MGH 78578 (KPMGH); *Pseudomonas entomophila* str. L48 (PSEEN); *Streptomyces avermitilis* MA-4680 (STRAV); *Arcobacter butzleri* RM4018 (ARCBU); *Azoarcus* sp. BH72 (AZOAR); *Pseudomonas syringae* pv. *Phaseolicola* 1448A CP000058 (PSESY); *Idiomarina loihiensis* L2TR (IDILO).

was elucidated, suggesting the involvement of sulfide oxidation (Pott and Dahl, 1998; Leloup *et al.*, 2006). ORF-25 showed significant amino acid sequence similarities with those of the sulfur oxidizers of the *Riftia pachyptila* endosymbiont (79%) (Robidart *et al.*, 2008) and *Thioalkalivibrio* sp. HL-EbGR7 (77%) (CP001339).

## Fosmid clones of carbon fixation and nitrate reduction genes

Other important genes that might be involved in energy metabolism and carbon fixation in the culture were analyzed from the metagenomic library. The existence of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Wildman, 2002; Portis and Parry, 2007; Feller *et al.*, 2008) gene - a key gene of the Calvin-Benson-Bassham cycle of $CO_2$ fixation - supports that the sulfur oxidizers in the enrichment culture are capable of autotrophic growth. We detected genes related to *cbbL* in fosmid clone 11G07 and *cbbM* in fosmid clone 12E11 and 12H01 of RubisCO. The two *cbbM* sequences are completely identical. *cbbL* sequences are broadly divided into

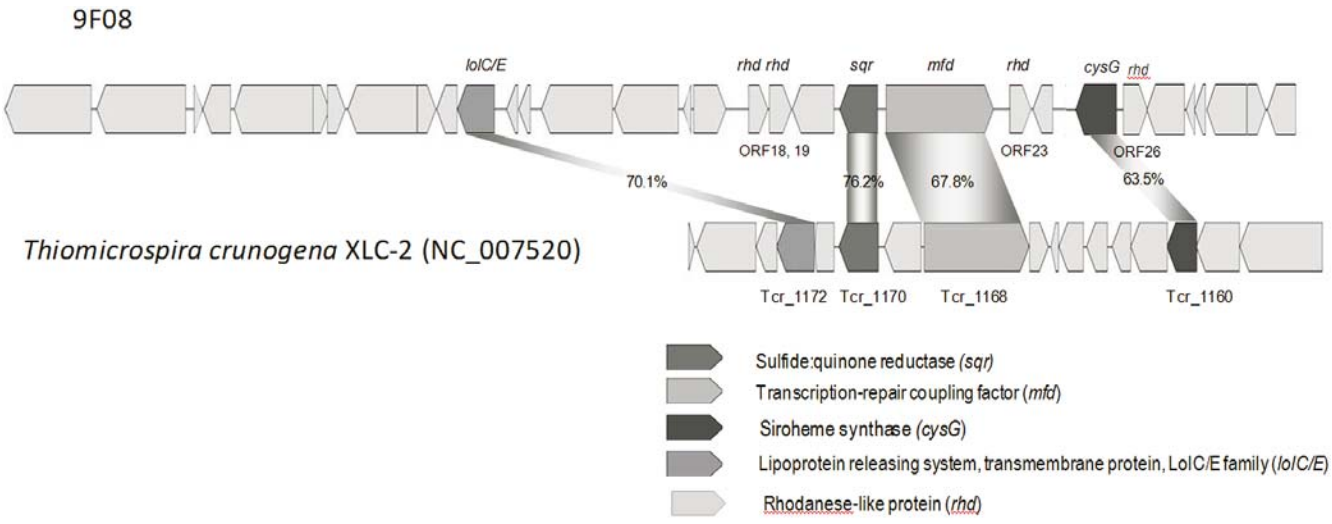


**Fig. 2.** Comparative gene organization of the *sqr*-containing clone 9F08 and *T. crunogena* XLC-2. Homologous regions were connected by shading. ORF numbers match those shown in Table 2 (*sqr*, ORF-11; *mfd*, ORF-22; *cysG*, ORF-25; *lolC/E*, ORF-11). The similarity percentage at the nucleotide level between the predicted gene products identified in the 9F08 clone and the genome of *T. crunogena* XLC-2 are shown within the corresponding shade regions (*sqr*, 76.2%; *mfd*, 67.8%; *cysG*, 63.5%; *lolC/E*, 70.1%). Genes indicated by Tcr followed by a number were obtained from the genome of *T. crunogena* XLC-2 (NC_007520).

two major groups, termed "green-like"and "red-like" (Delwiche and Palmer, 1996). The *cbbL* gene of fosmid clone 11G07 (cbbL-9) encoding the 'green-like' form I RubisCO large subunit was closely related to the uncultured prokaryote *cbbL* gene retrieved from the Suiyo Seamount Hydrothermal Vent (85.9% amino acid similarity) (Elsaied *et al.*, 2007) (Fig. 3A), whereas the *cbbM* (cbbM-3) encoding form II RubisCO had a close relationship with that of the *Thiobacillus* sp. '*Lamellibrachia* symbiont-1' (85.8% amino acid similarity) (Fig. 3B) (Elsaied and Naganuma, 2001). It has been suggested that the primary production of organic carbon at the Suiyo Seamount Hydrothermal Vent depends principally on sulfur-oxidizing bacteria (Elsaied *et al.*, 2007). All of these genes are related to those of the γ-proteobacterial sulfur oxidizers. It has also been reported that both *cbbL* and *cbbM* were present in *Thiomicrospira* species (Tourova *et al.*, 2006). The results of end-sequence analysis of the *cbbL*-containing clone 11G07 showed that one of the end sequences exhibited the best hit to the 50S ribosomal protein L19 (*rplS*) (GU362413) of *T. crunogena* XCL-2 (YP_390920), with an amino acid similarity of 74.7%, indicating that the genome fragment was of γ-proteobacterial origin.

The reduction of nitrate as an electron acceptor is a key energy-generating process in the denitrifying culture. As a molecular marker of denitrifying bacteria, the diversity of *narG* (a membrane-bound nitrate reductase gene) has been previously studied in the marine environment (Kandeler *et al.*, 2006; Smith *et al.*, 2007). We detected two clones (13D10 and 13D11) containing the nitrate reductase gene from the fosmid library. The two *narG* sequences were identical. No candidate dissimilatory nitrate reductase genes were available for comparison in the genome of *T. crunogena* XCL-2 (YP_390920) since the only electron acceptor they can use is oxygen. Although *T. microaerophila* ALEN1 was found to be closely related to 16S-6, nitrate did not support the anaerobic growth of the organism (Sorokin *et al.*, 2002). Phylogenetic analysis demonstrated that this *nar*G gene, narG-13, was the most closely related to that of *T. denitrificans* belonging to the β-proteobacteria (86.4% amino acid similarity) (Fig. 3C). It has been frequently noted that closely related variants of *narG* exist throughout distantly related taxa (Philippot *et al.*, 2002). This indicates that *narG* phylogenies are not consistently reflective of 16S rRNA phylogenies.

In conclusion, we characterized a sulfur-oxidizing enrichment culture via the metagenomic analysis of chemolithotrophic sulfur oxidizers enriched under denitrifying conditions. Based on the genome comparison of the *sqr*-containing fosmid clones, as well as the phylogenetic analysis of carbon fixation genes and the 16S rRNA gene, the phylotype of γ-proteobacteria was suggested to be the dominant sulfur-oxidizer in the enrichment culture. The γ -proteobacteria are closely affiliated with the sulfur-oxidizers detected in alkaline salterns and hydrothermal vents (Sorokin *et al.*, 2002; Elsaied *et al.*, 2007). In cold marine sediments, a large fraction of the microbial population is closely related to the γ-proteobacterial sulfur oxidizers (Ravenschlag *et al.*, 1999). Our metagenomic studies provide additional evidence to that sulfur-oxidizing γ-proteobacteria might be one of the important functional guilds in biogeochemical sulfur cycles in marine environments.

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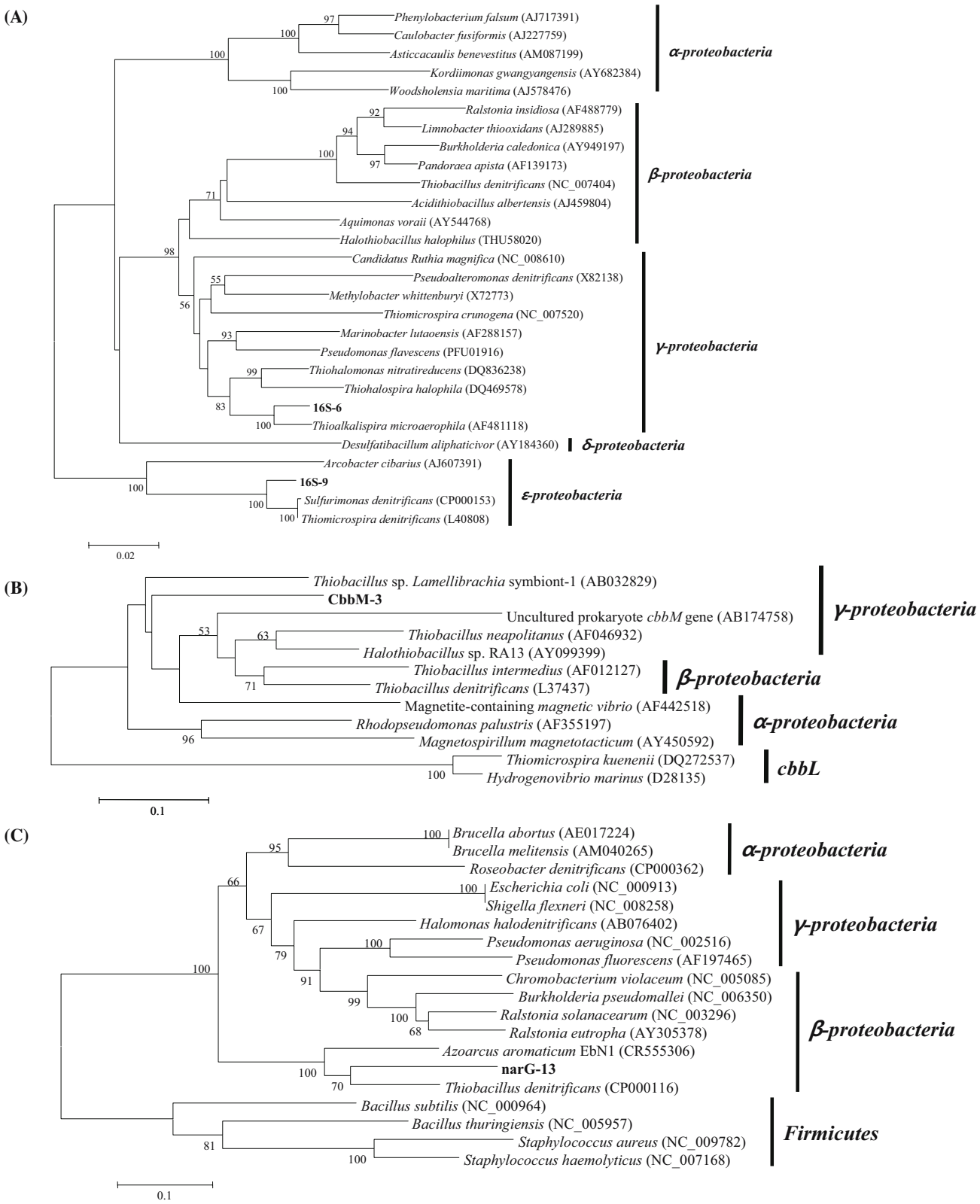


**Fig. 3.** Phylogenetic tree of (A) *cbbL* (about 770 bp), (B) *cbbM*, (about 350 bp), and (C) *narG* (about 690 bp) genes based on nucleotide sequences. Branching patterns supported by more than 50% bootstrap values (1,000 iterations) via neighbor-joining are indicated by their respective bootstrap values. Clones from this study are indicated in bold. The reference sequences were selected to indicate the relatives to the sequences determined in this study. The outgroup sequences of *cbbL* and *cbbM* were selected from *cbbM* and *cbbL*, respectively. The scale bar represents a 10% estimated sequence divergence.

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