Molecular analysis of the diversity of the sulfide : quinone reductase (*sqr*) gene in sediment environments

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Our newly designed primers were evaluated for the molecular analysis of specific groups of the *sqr* gene encoding sulfide : quinone reductase (SQR) in sediment environments. Based on the phylogenetic analysis, we classified the *sqr* sequences into six groups. PCR primers specific for each group were developed. We successfully amplified *sqr*-like gene sequences related to groups 1, 2 and 4 from diverse sediments including a marine sediment (SW), a tidal flat (TS), a river sediment (RS) and a lake sediment (FW). We recovered a total of 82 unique phylotypes (based on a 95 % amino acid sequence similarity cutoff) from 243 individual *sqr*-like gene sequences. Phylotype richness varied widely among the groups of *sqr*-like gene sequences (group 1 > group 2 > group 4) and sediments (SW > TS > RS > FW). Most of the *sqr*-like gene sequences were affiliated with the *Proteobacteria* clade and were distantly related to the reference *sqr* gene sequences from cultivated strains (less than ~80 % amino acid sequence similarity). Unique *sqr*-like gene sequences were associated with individual sediment samples in groups 1 and 2. This molecular tool has also enabled us to detect *sqr*-like genes in a sulfur-oxidizing enrichment from marine sediments. Collectively, our results support the presence of previously unrecognized *sqr* gene-containing micro-organisms that play important roles in the global biogeochemical cycle of sulfur.

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INTRODUCTION

Sulfide, the most reduced form of inorganic sulfur, occurs in sediment environments, where it is generated from anaerobic sulfate-reducing bacteria (Detmers *et al.*, 2001; Sorensen *et al.*, 1981). Besides chemical oxidation, micro-organisms are involved in the oxidation of sulfide at oxic–anoxic interfaces; this reaction initiates the first step of the sulfur oxidation process (Cytryn *et al.*, 2005; Jorgensen, 1982) and produces sulfate or sulfur as the major oxidation product (Friedrich, 1998; Ruepp *et al.*, 2000; She *et al.*, 2001; Theissen *et al.*, 2003).

The oxidation of sulfide to sulfate is mediated by membrane-bound electron-transport systems. Two main

enzymic systems participating in the initial step have been intensively studied to understand the mechanism of sulfide oxidation. The electrons from sulfide enter the electron-transport chain either at the level of *c*-type cytochromes, via a sulfide : cytochrome *c* reductase (FCC) (Kusai & Yamanaka, 1973a, b, c; Lyric & Suzuki, 1970a, b, c), or at the level of quinone via a sulfide : quinone reductase (SQR) (Nubel *et al.*, 2000; Schutz *et al.*, 1997, 1998, 1999). FCC was suggested to play an essential role in sulfide oxidation *in vivo*, and found in a number of chemotrophic and phototrophic sulfide-oxidizing bacteria (Griesbeck *et al.*, 2000; Schutz *et al.*, 1999). However, it is not obligatory in sulfide oxidation since FCC does not occur in a variety of sulfide-oxidizing bacteria, and appears to be dispensable in the micro-organisms in which it is present (Brune, 1995). For example, disruption of the *fcc* gene in *Chromatium vinosum* did not have any significant effect on the sulfide-oxidizing ability of the cells, showing that the gene was not essential for sulfide oxidation (Reinartz *et al.*, 1998).

The activity of SQR has been investigated in a diverse range of micro-organisms (Theissen *et al.*, 2003). This enzyme

Abbreviations: FW, freshwater lake sediment; RS, river sediment; SQR, sulfide : quinone reductase; SW, marine sediment; TS, tidal flat.

The GenBank/EMBL/DDBJ accession numbers for the partial *sqr*-like gene sequences determined in this study are EF506241–EF506485; the 16S rRNA gene accession numbers are EF541177–EF541178.

A supplementary figure and table are available with the online version of this paper.

was first isolated from thylakoids of the cyanobacterium *Oscillatoria limnetica* (Arieli *et al.*, 1994). Since then, SQR activity has been investigated in a variety of phototrophic and chemotrophic bacteria including *Chlorobium limicola* (Shahak *et al.*, 1992), *Paracoccus denitrificans* (Schutz *et al.*, 1998) and *Allochromatium vinosum* (Reinartz *et al.*, 1998) (for reviews see Griesbeck *et al.*, 2002; Shahak *et al.*, 1999). The *sqr* gene from the phototrophic bacterium *Rhodobacter capsulatus* has been isolated, sequenced, and expressed in *Escherichia coli* (Schutz *et al.*, 1997). Inactivation of the *sqr* gene causes the loss of phototrophic oxidation and growth capability of *R. capsulatus* with sulfide (Schutz *et al.*, 1999). Nubel's group investigated the sulfide oxidation capability of SQR in membranes of *Aquifex aeolicus*, one of the most hyperthermophilic and chemolithoautotrophic bacteria, and found that this gene is involved in sulfide-dependent respiration (Nubel *et al.*, 2000). A gene from the genome of *Aq. aeolicus* VF5 was identified to encode a protein with significant similarity to the SQR of *R. capsulatus* (Deckert *et al.*, 1998). Furthermore, archaeal and mitochondrial oxidation of sulfide is mediated by SQR (Friedrich, 1998; Ruepp *et al.*, 2000; She *et al.*, 2001; Theissen *et al.*, 2003). Diverse symbiotic sulfide-oxidizers have been reported to provide their hosts with carbon and a large array of additional nutrients by using the chemical energy from reduced sulfur (Cottrell & Cary, 1999; Duperron *et al.*, 2006). Consequently, SQR has been postulated as an enzyme potentially responsible for sulfide oxidation in wide variety of prokaryotes (Dahl *et al.*, 2002). And biological sulfide oxidation is important for the global circulation of sulfur and carbon in various oxic-anoxic interface environments (Griesbeck *et al.*, 2000).

Biological sulfide oxidation occurs in all three domains of life (Griesbeck *et al.*, 2002). Sulfide oxidation has been investigated in various eukaryotes (Furne *et al.*, 2001; Lee *et al.*, 1996; Vande Weghe & Ow, 1999; Yong & Searcy, 2001), including even humans (Goubern *et al.*, 2007), which are not specifically adapted to sulfide-rich environments. The activities of SQR and other enzymes allow the organism either to get energy (Oeschger & Vismann, 1994) or to detoxify sulfide entering the organism from the surrounding environment. This raises the possibility that sulfide oxidation is even more widespread among organisms, and might occur in a wider diversity of habitats; SQR is proposed as an ancient enzyme from the anaerobic world.

Molecular identification based on 16S rRNA genes does not necessarily correspond to metabolic function; therefore PCR techniques for various genes important in biogeochemical cycles have been developed for the functional study of microbial communities (e.g. *nirS*, *nirK*, *nosZ* and *narG* for denitrification) (Braker *et al.*, 2000; Kandeler *et al.*, 2006; Scala & Kerkhof, 1998; Smith *et al.*, 2007). Despite the importance of the sulfide oxidation step in sulfur biogeochemical cycles, development of a molecular tool for studying the diversity of the *sqr* gene involved in sulfide oxidation has not previously been attempted. In this study, we analysed the phylogeny of *sqr* gene sequences

retrieved from the GenBank database. We designed degenerate PCR primers targeting each group of *sqr* genes, and studied *sqr*-like gene sequence diversity in various sediment environments.

METHODS

Phylogenetic analysis of reference *sqr* sequences. To obtain the known sequences of the *sqr* gene, a nucleotide search of the GenBank database was performed using the keywords 'sulfide:quinone reductase', and the selected sequences were filtered manually. After a BLAST search using the selected *sqr* gene sequences, *sqr* homologues were retrieved again from GenBank. Finally 58 sequences were selected to analyse the phylogeny of *sqr* as of September 2006.

Sequence alignment was performed using the programs CLUSTAL W (Thompson *et al.*, 1994) and MAFFT (Niu *et al.*, 2006). The aligned results exhibited very low similarity to each other in both programs as follows: the mean *q* score, a sequence similarity scoring function, was 11.75 for CLUSTAL W and 12.48 for MAFFT (*q* score is scored from 0 to 100 for each aligned position). For comparison of phylogenetic trees of 16S rRNA gene and *sqr* sequences, the truncated regions of the aligned *sqr* sequences were eliminated to avoid the regions being treated as gaps. The alignment length including gaps of each group was as follows: group 1, 1372 nt and 447 aa; group 2, 1496 nt and 410 aa; group 3, 869 nt and 409 aa; group 4, 1470 nt and 485 aa; group 5, 1430 nt and 410 aa.

Before performing the phylogenetic analysis for all the sequences, we estimated the best-fit evolution model for those sequences using ProtTest (Abascal *et al.*, 2005), a program which evaluates the sequences for 80 combinations of protein evolution model following the Akaike information criterion (Posada & Buckley, 2004). The best model for the selected sequences was a WAG model for both alignments (CLUSTAL W and MAFFT). Due to the low sequence similarity, we selected two methods, maximum-likelihood and Bayesian inference, for phylogenetic analysis. Maximum-likelihood analyses utilized the program Tree-puzzle 5.2 (Schmidt *et al.*, 2002), and the WAG model allowing between-site rate variation was chosen for the protein evolution model. Bayesian analyses using the WAG model were performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Each inference starting from a random tree and using four Metropolis-coupled Markov Chain Monte Carlo runs (Altekar *et al.*, 2004; Beiko *et al.*, 2006) consisted of 100 000 generations with sampling every 100 generations.

Bacterial strains and genomic DNA isolation. The selected reference strains for validation of newly designed primers (listed in the legend of Fig. 2) were obtained from the DSMZ (Braunschweig, Germany; <http://www.dsmz.de>) as actively growing cultures. The genomic DNA from the reference strains was obtained by applying the Genomic DNA extraction kit (Solgent) according to the manufacturer's instructions. DNA concentration and purity were estimated by spectrophotometry (Nanodrop Technologies), and visual examination after electrophoresis on 0.8% (w/v) agarose gels.

Primer design and evaluation using reference strains. Several sets of degenerate PCR primers used to amplify *sqr* gene fragments were designed based upon each group of *sqr* gene sequences as classified above. The properties of the most successfully devised primer sets are listed in Table 1. To establish the new primers, 14 reference strains harbouring *sqr* genes were tested.

One microlitre (50 ng) of extracted genomic DNA was used as a template in 20 µl amplification reaction mixture. The *sqr* gene sequence was amplified using an AB 2720 thermal cycler (Applied

Table 1. Oligonucleotide primers designed and used in this study

Target	Primer name	Sequence (5'–3')	Position*	F or R†	T _m
Group 1	SQR-G1-475F	TGY TWY GGC CCG GCB TAY GA	475–494	F	59.8
	SQR-G1-964R	GTS ACC ATS SWT TCR ATC AT	964–984	R	52.3
Group 2	SQR-G2-145F	TGG ACC CTG GTG GGC GSS GG	145–164	F	67.7
	SQR-G2-490R	TTC WKC GGC GCG CCS SCG CA	490–509	R	66.7
Group 3	SQR-G3-199F	TBT AYS AGC CGG GWC TKC TBT	122–143	F	58.5
	SQR-G3-566R	GGY GCM ACS GGG CAT TTG	471–488	R	58.9
Group 4	Ge-140F	TGG ATT CCR TCR AAC ATT TGG GT	121–143	F	62
	Ge-840R	AAT WAG CAT MGC RAA RTC GAA CTC	823–846	R	57.4
Group 5	SQR-G5-437F	TTC AAA GGW GGA AMW ATW RC	445–464	F	51.3
	SQR-G5-799R	TKC CCR GTR TAY GGT GGM A	809–837	R	56
	SQR-G5-903R	CAT CRC CYA CWG CRT ARA CGT TAT C	913–937	R	60.6

*Position in the *sqr* gene of *Thiobacillus denitrificans* ATCC 25259 (NC_007404) from group 1, *Staphylococcus aureus* (NP_370612) from group 2, *Chlorobium tepidum* (NP_661917) from group 3, *Chlorobium tepidum* (AE006470) from group 4, and *Sulfolobus solfataricus* (NC_002754) from group 5, respectively.

†F, forward; R, reverse.

Biosystems) with *EF*-Taq DNA polymerase (Solgent). Group 1 *sqr* genes were amplified using the following PCR cycles: 5 min at 94 °C; 2 cycles of 30 s at 94 °C, 30 s at 45 °C, 30 s at 72 °C; 2 cycles of 30 s at 94 °C, 30 s at 50 °C, 30 s at 72 °C; 30 cycles of 30 s at 94 °C, 30 s at 52 °C, 30 s at 72 °C; 7 min at 72 °C; and hold at 10 °C. Group 2 *sqr* gene sequences were amplified using the following PCR cycles: 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 66.5 °C, 30 s at 72 °C; 5 min at 72 °C; and hold at 10 °C. Group 3, group 4 and group 5 *sqr* gene sequences were amplified using the same PCR cycles as for group 2 but with the elongation step at 58, 53 and 51 °C, respectively, instead of 66.5 °C.

Sample collection, DNA extraction and *sqr* gene amplification.

Sediment cores were obtained using a core sampler. Composite sediment samples were prepared by thoroughly mixing surface layer sediments from each site. Marine sediments (SW) were collected with a core sampler from a site in the East Sea, Korea, during July and August 2005. Sampling locations and water depth were as follows: ST3 (128°35' E, 38°20' N, 650 m). The water column above the sediments was oligotrophic, with typical ocean water. Surface samples of tidal flat (TS) were collected from a coastal area at Daechon, Korea (126°58' E, 36°53' N). We collected freshwater sediments (FW) from several metres water depth from the Daechung Reservoir in Korea during the summer bloom season (September 2005). The reservoir is a large branch-type artificial lake created by the construction of a 72 m-high dam in 1980. River sediment samples (RS) were collected from the Musimcheon River (127°28' E, 36°38' N), which flows through Cheongju city and is highly eutrophic. All samples were collected in sterile plastic tubes using alcohol-sterilized spatulas, and stored at –80 °C until analysis.

Total genomic DNA was extracted from 0.5 g of frozen sample using a Power Soil DNA extraction kit (Mo Bio Laboratories), following the manufacturer's instructions. We determined the total genomic DNA concentration using a spectrophotometer (Nanodrop Technologies). Samples with at least 30 ng DNA µl^{–1} were used for our experiments.

We amplified *sqr* gene sequences from the sediment DNA by following the optimized PCR conditions. Only amplicons of the expected size were excised from agarose gels to make libraries. Amplification products were purified using a PCR purification kit (Solgent), ligated into the T&A Cloning Vector kit (Real Biotech Corporation). *E. coli* DH5α cells were transformed with this ligated

vector according to the manufacturer's instructions. Putative positive clones were transferred to a 96-well plate containing Luria broth with ampicillin, grown overnight at 37 °C, and PCR-screened directly for the presence of inserts using the M13 universal primer set. PCR was performed under the following conditions: 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C; 5 min at 72 °C; hold at 10 °C. The positive clones from each library were randomly selected, purified using the PCR purification kit (Solgent) and sequenced directly using Bigdye terminator cycle sequencing ready reaction kits (Applied Biosystems).

Phylogeny and diversity analysis of environmental *sqr* sequences. Multiple alignments of environmental *sqr* sequences were performed by the same method as used to analyse the reference *sqr* sequences as described above. Phylogenetic trees were constructed by the maximum-likelihood method, using the program Tree-puzzle 5.2 (Schmidt *et al.*, 2002). Bootstrap analysis (500 replicates) was used to obtain confidence estimates for phylogenetic tree topologies.

Estimations of phylotype coverage, richness, evenness and diversity indices of *sqr* gene sequences were calculated with ESTIMATES (<http://viceroy.eeb.uconn.edu/EstimateS>). The species rarefaction curve of the entire dataset of each group was computed using the individual-based Coleman method with PAST (<http://folk.uio.no/ohammer/past>). The bias-corrected Chao1 estimator of phylotype richness was calculated after 1000 randomizations of sampling without replacement. The percentage of coverage was calculated by Good's method with the formula $[1 - (n/N)] \times 100$, where n is the number of phylotypes in a sample represented by one clone (singletons) and N is the total number of sequences in that sample (Good, 1953). Diversity of the sampled sequence set was estimated using the Simpson and Shannon indices within the ESTIMATES application. The Shannon index of evenness was calculated with the formula $E = e^{D/N}$, where D is the Shannon diversity index.

Enrichment of sulfur-oxidizing micro-organisms from marine sediments. Denitrifying enrichment cultures grown on thiosulfate were established with sediment samples of SW. One gram of sediment was inoculated into 100 ml of an anaerobic artificial marine salt medium (Fennell *et al.*, 2004) containing 1 mM of each sulfur compound as the sole electron donor, and 1 mM nitrate as the sole electron acceptor, in a 120 ml serum bottle. After inoculation, the bottle was purged with N₂ gas for 30 min and incubated at 15 °C in

the dark. The culture was maintained through successive serial dilutions for 3 months. The turbidity of the culture medium was indicative of thiosulfate oxidation and cell growth. An enrichment culture verified to oxidize tetrathionate, sulfite, sulfide, polysulfide and elemental sulfur in addition to thiosulfate was selected for further molecular analysis. In addition to turbidity, nitrate reduction (Rhee *et al.*, 1997) was used as an indicator of oxidation of sulfur compounds. *sqr* gene sequences were amplified using PCR conditions developed in this study. In order to analyse the microbial composition of this enrichment culture, we amplified the bacterial 16S rRNA gene sequences using primers 27F and 1492R (Weisburg *et al.*, 1991) and constructed a clone library (Park *et al.*, 2006). Sequences of the 16S rRNA gene obtained from the clone library were analysed as described previously (Park *et al.*, 2006).

RESULTS AND DISCUSSION

Phylogenetic analysis of *sqr* sequences

Aligned sequences were manually readjusted due to poor *sqr* sequence conservation in order to allow inter-group sequence comparison. When reference sequences were phylogenetically analysed and compared using the distance matrix, maximum-parsimony and maximum-likelihood methods, we classified the *sqr* sequences into six distinct

groups as shown in Fig. 1. Only one phylogenetic tree, Bayesian inference using MAFFT alignment, is illustrated. The presence of *sqr* sequences in diverse organisms suggests that sulfide oxidation by SQR is a ubiquitous trait in life that probably evolved from the early anoxic Earth (Theissen *et al.*, 2003).

We identified three of the SQR fingerprints suggested by Griesbeck *et al.* (2002) – Cys-159, FAD-binding domain III Gly-299 and Cys-353 – in five of the groups, as shown in Supplementary Fig. S1, available with the online version of this paper. Most of the other motifs and residues suggested by Griesbeck *et al.* (2002) were conserved, and the connecting fragment lengths were invariable. The universally conserved Cys-159 was replaced by Arg in the unclassified group; thus this group was excluded from further analysis. The average inter-group amino acid similarity of SQR sequences was approximately 24 %. The average intra-group amino acid sequence similarities of *sqr* groups 1, 2, 3, 4, and 5 were approximately 47, 39, 37, 66, and 61 %, respectively.

Phylogenetic trees were calculated from the 16S rRNA genes of identical organism sets harbouring each group of *sqr* gene sequences. Each *sqr* group contains specific

Fig. 1. Phylogenetic analyses of *sqr* sequences. Sequence groups are indicated to the right of the tree. Bootstrap values >50 (expressed as percentages of 100 replications) are shown at branch points. The scale bar indicates 0.2 substitutions per site.

phylogenetic groups: group 1, *Cyanobacteria* and *Proteobacteria*; group 2, *Firmicutes* and *Proteobacteria*; group 3, *Archaea* and others; group 4, ϵ -*Proteobacteria* and green sulfur bacteria; group 5, *Archaea*. In groups 1 and 2, which contain the majority of reference *sqr* sequences, we found a rough correlation in topology of the phylogenetic trees between *sqr* and 16S rRNA gene sequences. This result indicates that the possibility of promiscuous lateral *sqr* gene transfer could be excluded.

Design and evaluation of PCR primers for amplification of *sqr* gene sequences

Due to the high diversity of *sqr* gene sequences, we could not find any regions that were sufficiently highly conserved to design universal PCR primers covering all of the *sqr* groups. Instead, DNA sequences from each *sqr* group were aligned and used to screen priming sites for PCR amplification. We found several conserved regions for designing primers in each group (Table 1).

For evaluation of the primers designed, we obtained representative reference strains known to harbour genes of each *sqr* group from the DSMZ microbial collection. Using the genomic DNA as PCR template, PCR amplification conditions for the newly designed primers were tested. *sqr* gene sequences from reference strains were specifically amplified and yielded PCR products of the expected length (Fig. 2). PCR amplification yielded amplicons of approximately 500 bp, 360 bp, 360 bp, 720 bp, and 390 bp plus 490 bp for groups 1, 2, 3, 4 and 5, respectively. The sequences of PCR amplicons were confirmed by sequencing the band extracted from the gel.

Diversity analysis of environmental *sqr* sequences

Diverse sulfide-oxidizing micro-organisms have been isolated from a wide variety of oxic–anoxic interface environments (Brune, 1995). However, the cultivation-independent molecular diversity of sulfide-oxidizing micro-organisms could not be studied. We successfully amplified *sqr*-like genes using the primer sets designed for groups 1, 2 and 4 from most of the sediment samples. PCR amplification using primers targeting *sqr* groups from extremophilic *Archaea* (groups 3 and 5) failed despite further PCR optimization efforts.

An extensive PCR survey of *sqr* gene sequences using our primers resulted in the amplification of diverse *sqr*-like gene sequences both from freshwater and marine sediment environments (Fig. 3). The *sqr*-like gene sequences retrieved from these environments exhibited an average amino acid sequence similarity of approximately 70, 64, and 75 % in group 1, 2, and 4, respectively. Motifs and spacing between these motifs were conserved in the *sqr*-like gene sequences recovered from sediments. The phylogenetic trees constructed for each group using the *sqr*-like gene sequences are shown in Fig. 4(a, b, c). Our results suggest that these micro-organisms are diverse and ubiquitous in sediment environments.

We recovered a total of 82 unique phylotypes (group 1, 44; group 2, 29; group 4, 9) based on a 95 % amino acid sequence similarity cut-off from 243 individual sequences. Estimations of species coverage, richness, evenness and diversity indices were calculated for each group, as well as for four subsets of sediment samples (Supplementary Table

Fig. 2. Agarose gel electrophoresis showing amplification products from pure cultures using the degenerate primers designed in this study. Lane numbers indicate size markers or amplicons from reference strains as follows: M, molecular size markers (100 bp ladder); 1, *Rhodobacter capsulatus* (DSM 155); 2, *Thiobacillus denitrificans* (DSM 12475); 3, *Thiomicrospira crunogena* (DSM 12353); 4, *Rhodobacter sphaeroides* (DSM 158); 5, *Cupriavidus metallidurans* (DSM 2839); 6, *Chloroflexus aurantiacus* (DSM 635); 7, *Chlorobium tepidum* (DSM 12025); 8, *Magnetospirillum magnetotacticum* (DSM 3856); 9, *Archaeoglobus fulgidus* (DSM 4304); 10, *Pelodictyon luteolum* (DSM 273); 11, *Thiomicrospira denitrificans* (DSM 1251); 12, *Prosthecochloris aestuarii* (DSM 271); 13, 15, *Thermoplasma acidophilum* (DSM 1728); 14, 16, *Sulfolobus solfataricus* (DSM 1616). Two reverse primers were tested to amplify *sqr* gene sequences from the reference strains of group 5 (lanes 13–16).

Fig. 3. Agarose gel electrophoresis of PCR products from sediment samples using the degenerate primers designed in this study. The name of the sample is indicated above each lane. M, molecular size markers (100 bp ladder). PCR products of each predicted size are marked by arrows.

S1). Phylotype richness varied widely among *sqr* groups (group 1 > group 2 > group 4) and sediment samples (SW > TS > FW > RS). These are likely to be minimal estimates because the rarefaction curve of groups 1 and 2 did not plateau with the current sequencing effort (data not shown). Conventional diversity and evenness indices were also lowest for group 4.

Based on the phylogenetic analysis of PCR clone libraries, we found that individual sediment samples often had unique *sqr*-like gene sequences associated with them (Fig. 4), i.e. clades of unique *sqr*-like gene sequences clustered according to habitat. Interestingly, no environmental clone sequence was closely related to those of cultured relatives. This indicates that we have been underestimating the diversity of *sqr* gene sequences that may be involved in sulfur biogeochemical cycles in sediment environments.

Group 1

The phylotype richness of group 1 is the highest among our environmental *sqr* groups. Furthermore, since we detected group 1 sequences in all our marine and freshwater sediments, this group may be abundant and play an important role in sulfide oxidation in oxic–anoxic interface environments; however, this needs to be supported by further investigation. Although the reference *sqr* gene sequences for group 1 are composed of those from *Proteobacteria* and *Cyanobacteria* (see Fig. 1), all of our *sqr*-like gene sequences were more closely related to those of *Proteobacteria* than *Cyanobacteria*. We could not assign our *sqr* gene sequences to a specific class of *Proteobacteria* since most of them are distantly related to current GenBank database sequences. The majority (40%) of RS clone sequences (representative clones, G1RS16 and G1RS20) were related to *sqr* gene sequences from a strain of β -*Proteobacteria*; these clones exhibited an amino acid sequence similarity with *Polaromonas naphthalenivorans* of 81 and 74 % respectively. The majority (32 %) of our *sqr*-like

gene sequences (representative clone G1FW4) from the FW environment were related to *sqr* gene sequences from a strain of γ -*Proteobacteria*; this clone exhibited an average amino acid sequence similarity of 83 % with *Methylococcus capsulatus*. Most of the sequences in this group were habitat-specific since there were only two shared phylotypes among the libraries from different sediments.

Group 2

Within group 2, we could only amplify diverse *sqr*-like gene sequences from SW and TS environments. Unique *sqr*-like gene sequences were associated with each habitat since no shared phylotypes were detected between these two sediments. Interestingly, we could detect only one phylotype (with a representative clone, G2FW2) from the freshwater sediments and no PCR amplification could be achieved from the river sediments. Most of the *sqr*-like gene sequences were related to those of *Proteobacteria*. Only one clone (G2TS21) was distantly related to the other sequences and was possibly affiliated with *Firmicutes*. In this group, the closest cultivated relatives still exhibited less than 74 % amino acid sequence similarity to environmental *sqr*-like gene sequences.

Group 4

The phylotype richness of group 4 was lower than that seen in other groups. With the exception of FW, most of the *sqr*-like gene sequences in this group were shared among sediment samples (with three shared phylotypes among nine phylotypes). The closest cultivated relative of most SW, TS and RS clones is *Thiomicrospira denitrificans* (ϵ -*Proteobacteria*), although these clones have less than 76 % amino acid sequence similarity with this ϵ -proteobacterial strain. Recently, ϵ -proteobacterial sulfide oxidizers were suggested to be important for sulfur biogeochemistry in sulfidic environments (Campbell *et al.*, 2006); however, since only one reference *sqr* gene sequence was available in this class of *Proteobacteria*, we could not identify the exact affiliation of our *sqr*-like gene sequences.

Fig. 4. Phylogenetic tree showing the affiliations of *sqr* gene sequences to selected reference sequences of each group. *sqr* gene sequences affiliated with group 1 (a), group 2 (b) and group 4 (c) are shown. The tree was calculated by maximum-likelihood analysis. Numbers of clones within each phylotype (<95 % sequence similarity as cutoff of phylotype) are shown in parentheses. Significant bootstrap values (≥ 50 %; 500 replications) are indicated by filled circles at branch points. The scale bar represents 20 % estimated sequence divergence (20 substitutions per 100 amino acids). The right side of the figure shows the relative abundance of phylotypes per samples in grey values (white, 0 % present; black, 100 % of clone library). Letters above the abundance graph correspond to environmental samples. Columns labelled 'Sum' considered all clones in each group. Clones marked with an asterisk (G1DH17 and G4DH2) are from the enrichment culture.

Detection of *sqr* genes in a sulfur-oxidizing enrichment culture

A sulfur-oxidizing enrichment culture from marine sediments was characterized using the primers developed in this study. The enrichment was highly purified after being transferred three times using dilution-to-extinction culture methods (see Methods). Only two major 16S rRNA gene sequences were detected in the clone library analysis. These 16S rRNA gene sequences (EF541177 and EF541178) were affiliated with free-living or symbiotic γ -proteobacterial sulfur oxidizers that were reported by Ravenschlag *et al.* (1999) (with 84 % 16S rRNA gene sequence similarity to cultured relatives) and *Thiomicrospira denitrificans* (Brettar *et al.*, 2006) (97 % 16S rRNA gene sequence similarity). We failed to isolate these micro-organisms since they could not develop colonies on any tested solid media.

One phylotype of the *sqr*-like gene sequences from groups 1 and 4 was amplified (indicated in Fig. 4a and c). The clone, G4DH2, from group 4 is closely related to *Thiomicrospira denitrificans* (Brettar *et al.*, 2006) (ϵ -*Proteobacteria*) with 89 % amino acid sequence similarity. The group 1 clone G1DH17 was distantly related to other environmental clones and cultured relatives, although it is loosely affiliated with those of γ -*Proteobacteria* (an endosymbiont, '*Candidatus* *Ruthia magnifica*', and *Thiomicrospira crunogena*) (Newton *et al.*, 2007; Scott *et al.*, 2006). In cold marine sediments, many clones (18 %)

are closely related to symbiotic and free-living γ -proteobacterial sulfur oxidizers (Ravenschlag *et al.*, 1999). These results indicate that the enriched strain could be one of the sulfide oxidizers playing important roles in sulfur biogeochemical cycles in marine sediment environments. In this study, we could detect and tentatively correlate our novel *sqr* gene sequences with 16S rRNA gene sequences in the enrichment culture. Our results demonstrated that the molecular tools we have developed might be successfully applied to the characterization of diverse micro-organisms involved in sulfide oxidation.

Although the majority of the *sqr*-like gene sequences retrieved from various sediments were related to those of diverse *Proteobacteria*, we could not find any *sqr* gene sequences closely related to those of cultivated micro-organisms. This indicates that the micro-organisms containing these *sqr*-like gene sequences are previously undiscovered, and diverse micro-organisms involved in sulfide oxidation still remain to be investigated. Further work is necessary to determine the phylogenetic position of these environmental *sqr* gene sequences.

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