

# Diversity and Composition of Sulfate- and Sulfite-Reducing Prokaryotes as Affected by Marine-Freshwater Gradient and Sulfate Availability

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**Abstract** Sulfate- and sulfite-reducing prokaryotes (SSRP) communities play a key role in both sulfur and carbon cycles. In estuarine ecosystems, sulfate concentrations change with tides and could be limited in tidal freshwater reach or deep sediments. In a subtropical estuary of northern Taiwan in December 2007, we examined the compositional changes of SSRP communities. We examined three sites: from the lower estuarine brackish-water reach (site GR and mangrove vegetation site, GM) to the upper estuarine tidal freshwater reach (site HR), as well as from surface to a 50-cm depth. The partial sequence of sulfite reductase (*dsrB*) genes was used as a molecular marker of SSRP, linked to polymerase chain reaction and denaturing gradient gel electrophoresis (DGGE) techniques. SSRP communities of the DGGE profiles varied with sites according to one-way analyses of similarities (Global  $R=0.69$ ,  $P=0.001$ ). Using cluster analysis, the DGGE profile was found to show site-specific clusters and a distinct depth zonation (five, six, and two SSRP communities at the GM, GR, and HR sites, respectively). SSRP composition was highly correlated to the combination of salinity, reduced sulfur, and total organic carbon contents (BIO-ENV analysis,  $r_s=0.56$ ). After analyzing a total of 35 *dsrB* sequences in the DGGE gel, six groups with 15 phylotypes were found, which were closely related to marine-freshwater gradient. Moreover, sequences neighboring

sulfite-reducing prokaryotes were observed, in addition to those affiliated to sulfate-reducing prokaryotes. Four phylotypes harvested in HR resembled the genus *Desulfotobacterium*, a sulfite-reducing prokaryote, which failed to use sulfate as an electron acceptor and were active in freshwater and sulfate-limited habitat. The other five phylotypes in the HR reach belonged to the sulfate-reducing prokaryotes of the genera *Desulfatiferula*, *Desulfosarcina*, *Desulfovibrio*, and *Desulfotomaculum*, which appeared to tolerate low salinity and low sulfate supply. SSRP phylotypes at the mangrove-vegetated GM site (five phylotypes in two groups) were phylogenetically less diverse, when compared with those at the non-mangrove-vegetated GR site (three phylotypes in three groups) and the tidally influenced freshwater HR site (nine phylotypes in five groups). Phylotypes found at GR and GM were all affiliated to marine sulfate-reducing prokaryote strains of the genera *Desulfofaba*, *Desulfobotulus*, *Desulfatiferula*, *Desulfosarcina*, and *Desulfotomaculum*. Notably, a phylotype recorded in the surface sediment at GR resembled the genus *Desulfobulbus*, which was recorded from freshwater environment consisting of the freshwater input at GR during ebb tides.

## Introduction

The dissimilatory reduction of sulfate is a key process in global sulfur cycle and organic matter mineralization in marine and freshwater ecosystems [4, 26, 43]. Sulfate reduction is mainly mediated by a phylogenetically and physiologically diverse group of microorganisms, called sulfate-reducing prokaryotes (SRP), which use sulfate as terminal electron acceptor in oxidizing various types of carbon sources [55]. SRP are a group of obligate anaerobic

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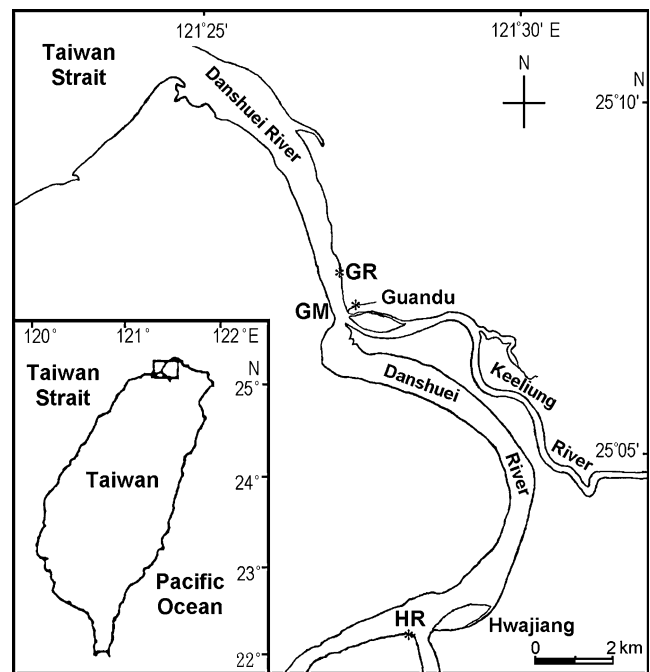
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prokaryotes, taxonomically belonging to five bacterial and two archaeal phyla [5, 18]. Besides the extremely anoxic environments, SRP are also discovered in various environments exposed to temporarily oxic condition including biofilms [53], microbial mats [35], and wastewater treatment systems [33].

Estuarine surface sediment exhibits temporarily oxic, hypoxic, or anoxic conditions with tidal inundation and in which sulfate supplied from the sea will be diluted by freshwater from upstream [42, 66]. Organic matter contributed from human activities besides the river and riparian vegetation also influences the carbon supply to the estuary [28, 66]. Several studies, however, demonstrated that at least certain SRP tolerate a high concentration of oxygen while some SRP, possessing metabolic versatility, can survive in places with a shortage of sulfate [33, 35, 55]. Understanding the composition of SRP communities and which abiotic factors influence their distribution is crucial as natural functions of degraded estuaries need to be restored. Limited data are available on SRP distributional patterns in estuarine wetlands where sulfate concentrations and sediment oxygenation vary constantly with tidal rhythm [48] and with increasing anthropogenic pollution threats.

During the last decades, by using 16S *rRNA* or functional genes as molecular markers, many new SRP taxa have been recorded [9, 20, 52]. Highly conserved functional genes, such as *dsrAB* and *aprBA*, were also applied for SRP, sulfite-reducing prokaryotes (SiRP), or sulfide-oxidizing prokaryotes (SOP) [25, 37, 46, 65]. Dissimilatory sulfite reductase, encoded by *dsrAB* gene, is a key enzyme which catalyzes the conversion of sulfite to sulfide [37, 65]. Its corresponding sequence of *dsrAB* genes provides a basis for culture-independent molecular diversity studies of natural assemblages with sulfite-reducing ability, mainly possessed by SRP and SiRP, while using polymerase chain reaction (PCR) primers broadly specific for a large fragment of all known *dsrAB* genes [16, 36, 37, 49, 65]. In addition, the phylogeny of *dsrAB* has shown that SOP having oxidative-operating sulfite reductase are monophyletic and distantly related to the SRP or SiRP which use a reductive type of enzyme [46].

Sulfate and organic matter are essential compartments for dissimilatory SRP growth, and therefore, are important factors controlling their distribution [55]. When the sulfate concentration is low, such as in freshwater environments, SRP is also capable of fermentation and anaerobic oxidation of organic compounds [39, 51]. In the Danshuei River estuary, northern Taiwan (Fig. 1), sea water provides high levels of sulfate to the lower estuary. Toward its upstream reaches, sulfate levels gradually decrease and eventually reach almost zero in tidally influenced freshwater area [29, 44]. Nevertheless, compared with that in the



**Figure 1** Study sites in the Danshuei River estuary, northern Taiwan. GR: brackish-water reach in the lower estuary in Guandu area; GM: mangrove-vegetated habitat in Guandu area; HR: tidal freshwater reach in the upper estuary in Hwajiang area

lower estuary, the sulfate-reducing rate in the upper freshwater area was markedly 200-fold higher [11]. Apparently, some unique SRP may be thriving in the sulfate-depleted upper reach. Decaying vegetative debris is a source of organic matter in the mangrove-vegetated habitat in the lower part of this estuary [27, 28]. Previous studies have also demonstrated the importance of redox conditions in affecting the distribution of SRP assemblages [7]. In addition to the supply of organic matter, mangrove is known to influence sediment redox condition due to oxygenation activity surrounding their root tips [41]. Moreover, pore-water sulfate level decreased with depth in the sediment of the lower reach estuary of the Danshuei River [21], while sediment redox recorded in intertidal mud flats also decreased as depth increased [56]. In the present study, we hypothesized that in the Danshuei River estuary, northern Taiwan, (1) SRP communities and gene types differ between tidal freshwater site and lower estuarine sites; (2) SRP communities and gene types differ between mangrove-vegetated and non-vegetated habitats in the lower estuarine area; and (3) their distributions exhibit depth zonation in sediments. We also examined the presence of other *dsrAB* gene evolving prokaryotes, i.e., SiRP and SOP, in the estuarine system. In this study, molecular fingerprints based on *dsrB*-targeted denaturing gradient gel electrophoresis (DGGE) were used as a proxy to describe the diversity of SRP. In addition, environmental

factors were also measured simultaneously. Finally, we discussed the possible factors controlling the presence of SRP in an estuary along the gradient from brackish-water to freshwater.

## Materials and Methods

### Environmental Samples

Spatial distributions of SRP were studied, and abiotic factors in the ambient were measured simultaneously by taking pair-sets of sediment cores from three sites in the Danshuei River estuary, northern Taiwan (Fig. 1). In the riverside, along a decreasing salinity and sulfate gradient from the lower toward upper estuary, two sites were selected, one in the lower estuarine brackish-water locality Guandu, GR, with salinity ranging 5.0–22.0 [29] and the other in the upper tidally influenced freshwater locality Hwajiang, HR, with nearly no salinity. The biogeochemical characteristics revealed that reduced sulfur compound in Guandu sediment were two- to fourfold higher than those in Hwajiang [21]. Inversely, sulfate-reducing rates were 200-fold lower ( $0.0654 \text{ Mday}^{-1}$  in Guandu vs.  $13.1 \text{ Mday}^{-1}$  in Hwajiang, 11). The third site was located also in the lower estuarine Guandu reach but within a mangrove-vegetated area, GM. This vegetation site was chosen in determining whether the existence of vegetation alters the microbial communities.

Each of the three pair-sets of the sediment samples was collected using PVC corer of 10 cm in diameter. During the low tide in December 2007, corers were pushed down approximately 1 m, sealed immediately after collection, and brought to the laboratory within an hour. Sediment cores were cut every 10 up to 50 cm in depth to collect six 1–2-cm thick subsamples. Subsamples for abiotic measurements were done in an argon-filled anti-oxidant bag to prevent the reaction of sulfur compounds with oxygen. These subsamples were then sealed in 50-ml centrifuge test tubes and refrigerated at  $4^\circ\text{C}$  until further treatment. Subsamples for microbial community analysis were sealed in 1.5-ml microtubes and stored at  $-80^\circ\text{C}$  until the environmental DNA were extracted within 2 days.

### Measurements of Abiotic Factors

Sediment samples were centrifuged at 4,000 rpm for 15 min. The supernatant was filtered through a  $0.45\text{-}\mu\text{m}$  Nuclepore filter to obtain pore-water. Salinity was determined using a refractometer (S/Mill-E, ATAGO, Tokyo, Japan). Sulfate was collected by precipitating dissolved sulfate with barium chloride ( $\text{BaCl}_2$ ) in a solution with pH lower than 2. Sulfide was measured using the methylene

blue method [15]. Total organic carbon (TOC) content was determined with a CHN analyzer (Perkin Elmer EA 2400 Series II; Wellesley, MA) after sediment samples had been freeze-dried and acid-fumed. Reduced sulfur (acid-volatile sulfur (AVS) and pyrite-S combined) was extracted and determined using diffusion method [30].

### Extraction and Purification of DNA

Approximately 1.0 g wet weight of sediment was taken for DNA extraction and purification using the UltraClean Soil DNA kit (MoBio, Solana Beach, CA) according to the manufacturer's instructions. Two microliters of nucleic acid extracts were mixed with  $5\times$  bromophenol blue loading buffer (50% [ $v/v$ ] glycerol, 50 mM Tris/HCl pH 7.5, 5 mM EDTA, 0.05% [ $w/v$ ] bromophenol blue), electrophoresed on 1% [ $w/v$ ] agarose (Invitrogen, Carlsbad, CA, USA) containing  $0.5 \mu\text{g ml}^{-1}$  ethidium bromide (Sigma, Saint Louis, MO), with  $1.0\times$  Tris–borate EDTA buffer [57]. Ethidium bromide stained bands were digitally recorded with an ImageQuant 300 Imager system (Amersham Bioscience, Buckinghamshire, UK). Quantification of the extracted DNA was performed by comparing to 100 bp DNA Ladder quantification standard (Geneaid, Taipei County, Taiwan). The analysis of the digital images was carried out using 1D gel analysis in the software package of ImageQuant TL (Amersham Bioscience, Buckinghamshire, UK).

### PCR Amplification of Partial *dsrB* Gene

PCR amplification of  $\sim 350$ -bps *dsrB* fragment was performed using the primers DSRp2060F and DSR4R [24, 65, 69], to which a 40-bps GC-clamp [50] was added at the 5'-end of the forward primer. Positive controls, as pretested sample from hypoxic sediment, and negative controls with blank were always included in PCR amplification experiments. The reaction was carried in a PxE Thermal Cycler (Thermo Electron Corp., Milford, MA). The 50- $\mu\text{l}$  reaction mixture contained 100 ng of soil DNA,  $1\times$  Optimized DyNAzyme<sup>TM</sup> EXT Buffer,  $0.5\text{-}\mu\text{M}$  concentrations of each primer,  $200\text{-}\mu\text{M}$  concentrations of each deoxynucleotide, and 1 U of DyNAzyme EXT DNA Polymerase mix. Thermal cycling was followed as described in Geets et al. [24]. The presence and size of the amplification products were determined by agarose (1.5% [ $w/v$ ]) gel electrophoresis.

### DGGE of *dsrB* Genes

Community compositions, expressed as band patterns, based on *dsrB* genes were generated using *dsrB*-based DGGE. A 1-mm-thick 8% polyacrylamide gel with a

denaturing gradient consisting of 40% to 70% urea-formamide was used for DGGE. Gels were ran at 60°C for 16 h at 80 V in 1× Tris–acetate–EDTA (TAE) buffer using a Bio-Rad Dcode universal mutation detection system (Bio-Rad Laboratories, Mississauga, ON, Canada). Following electrophoresis, the gels were incubated for 20 min in 1× TAE buffer containing 1× SYBR Gold nucleic acid gel stain (Molecular Probes Europe BV, The Netherlands) and photographed under UV light using an ImageQuant 300 Imager system with ImageQuant TL software (Amersham Bioscience, Buckinghamshire, UK).

#### Statistical Analysis of DGGE Profile

High-resolution digital DGGE images were analyzed using ImageQuant TL software. For data analysis, the images were normalized using the AlphaEase FC software, version 6.0 (Alpha Innotech, San Leandro, CA); the bands were detected using the band-searching algorithm of the software. After normalization of the gels, only the bands with a peak height intensity exceeding 5.0% of the strongest band in each lane were included in further analyses. Analysis of band patterns was performed with the Dice coefficient using 1% position tolerances and 0.5% optimization for the band migration distance. Clustering of patterns was performed by UPGMA dendrogram with a similarity matrix of Pearson coefficient. The stability of the dendrograms was evaluated by randomizing the sample order 100 times and recalculating the dendrograms with 95% background noise. The percentage of similarity between band patterns is used only to address the relatedness among the DGGE patterns and is not an indication of quantitatively genetic relatedness among respective samples.

The differences of DGGE profile patterns among sampling settings were examined using one-way analyses of similarities (ANOSIM) based on the similarity matrices [13]. Four settings were examined: (1) riverside locality at Guandu and Hwajiang, (2) vegetated and non-vegetated habitats at lower estuarine Guandu, (3) three sites, GM, GR and HR, and (4) six depths.

The relationships between six abiotic factors (salinity, and concentration of sulfate, sulfide, and reduced sulfur, as well as TOC content and sampling depth) and DGGE profile patterns were determined using BIO-ENV routine. BIO-ENV uses generalized Mantel test to examine associations between biotic and abiotic data expressed as Spearman rank correlation coefficients [13]. The matrices of DGGE profile patterns were directly come from the aforementioned cluster analysis, and the abiotic-factor matrices were based on Euclidean distance [14]. ANOSIM and BIO-ENV analyses were performed using PRIMER version 5.23 software package [14].

#### Sequence of *dsrB* DNA Fragments

A total of 35 DGGE bands including nine bands from site GR, 17 bands from site HR, and nine bands from site GM were excised and sequenced. Bands excised from the DGGE fingerprints were dissolved in 20 µl of deionized H<sub>2</sub>O and incubated overnight at 4°C in order to elute the DNA from the gel. Five microliters of the solution was used in a PCR reaction with the corresponding GC-clamped primers. These amplicons were purified with a Clean Up-M kit (Viogene) as specified by the manufacturer. Using dye terminator chemistry, DNA sequences were determined by direct sequencing with a DNA sequencer model 3730 (Applied Biosystems, Foster City, CA). The primer used for sequencing was DSR4R. The sequences obtained were compared with public database of amino acid sequences using BLAST to determine their potential taxonomic affiliation [2]. These sequences have been deposited in the NCBI nucleotide sequence databases under accession numbers HQ413099–HQ413133.

#### Phylogenetic Affiliation Analysis

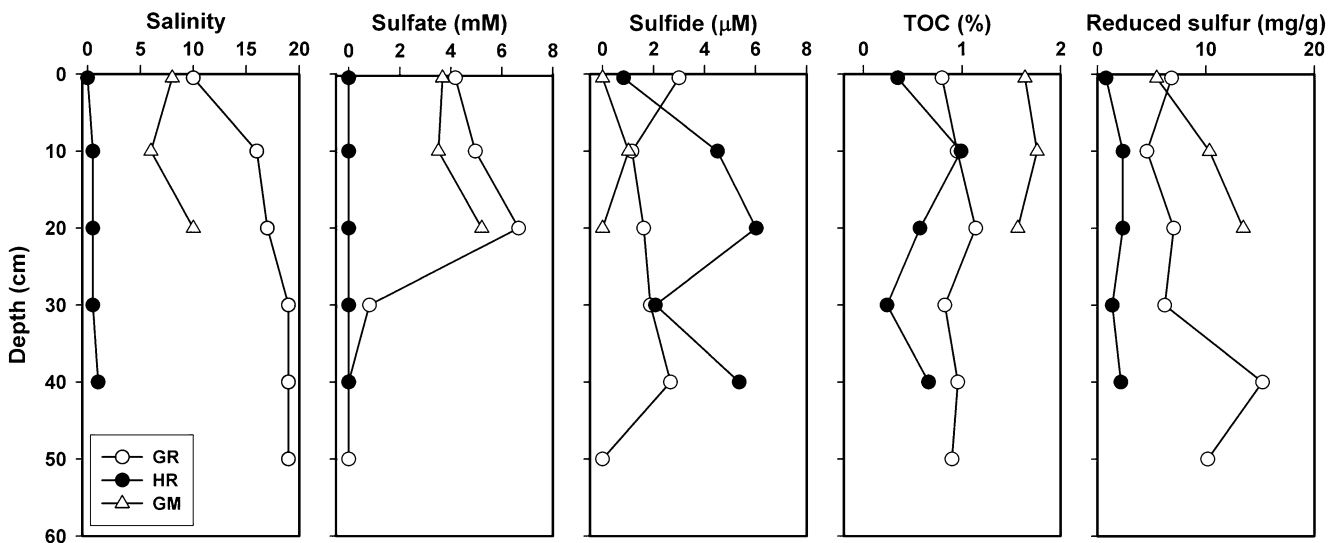
The *dsrB* gene sequences were aligned as amino acid sequences before being aligned against their closest relatives. Forty-seven sequences were chosen from the database [63], including 14 SRP, 3 SiRP, 9 SOP, and 21 environmental sequences. Phylogenetic analyses were performed using MEGA version 5.0 [60, 61]. Only unambiguously aligned positions were used. The *dsrB* matrices were analyzed by distance-matrix of neighbor-joining (NJ) [58], maximum parsimony (MP) [23], and maximum likelihood (ML) [22]. Here, 1,000 bootstraps were performed for the NJ, MP, and ML algorithms to analyze the relatedness of the *dsrB* genes. Multifurcations were created at the appropriate basal node when branching patterns were only supported in greater than 70% of the bootstrap resamplings. In this phylogenetic tree, the lowest determined taxon whose sequences were grouped within a concordant branch is termed a “phylotype” hereafter to indicate the taxonomic identification of our *dsrB* sequences.

## Results

### Abiotic Characteristics

All the abiotic factors measured exhibited site differences, while the differences were less for depth zonation in the Danshuei River estuary (Fig. 2). Pore-water salinity decreased from the lower estuarine brackish-water reach to the upper tidal freshwater reach in the range of 0 at HR to a high of 19 at GR. Sedimentary salinity in the two brackish-water





**Figure 2** Vertical distributions of salinity, concentrations of sulfate and sulfide, and total organic carbon (TOC) content and reduced sulfur concentration at site GR, GM, and HR

sites, particularly in GR, increased with depth. The salinities at the GM site were lower than those at GR, and at GM, a minimum of 6.0 appeared at the top 10-cm layer (Fig. 2). Pore-water sulfate concentration also decreased from the brackish-water reach to the tidal freshwater reach, ranging from 0 to 6.7 mM in the lower estuary to a constant 0 mM in the upper estuary. At both brackish-water GR and GM sites, the sulfate concentrations peaked at the depth of 20 cm below the surface, with values of 6.7 and 5.2 mM, respectively, and then sharply decreased to zero in further deep layers (Fig. 2). Comparatively, the pore-water sulfide concentrations at HR were higher than those at GR and GM, while GM had the lowest levels (2.1–6.0  $\mu\text{M}$  at HR vs. 0–3.0  $\mu\text{M}$  at GR and 0–1.0  $\mu\text{M}$  at GM, Fig. 2). TOC contents at GM ranged from 1.2% to 1.8% (weight-to-weight) and were higher than those at GR and HR with TOC contents generally  $<1.0\%$  (Fig. 2). Reduced sulfur contents decreased from the brackish-water reach to the tidal freshwater reach, with ranges from 4.6 to 15.2 and 0.8 to 2.3  $\text{mg g}^{-1}$ , respectively (Fig. 2).

#### Composition of SRP

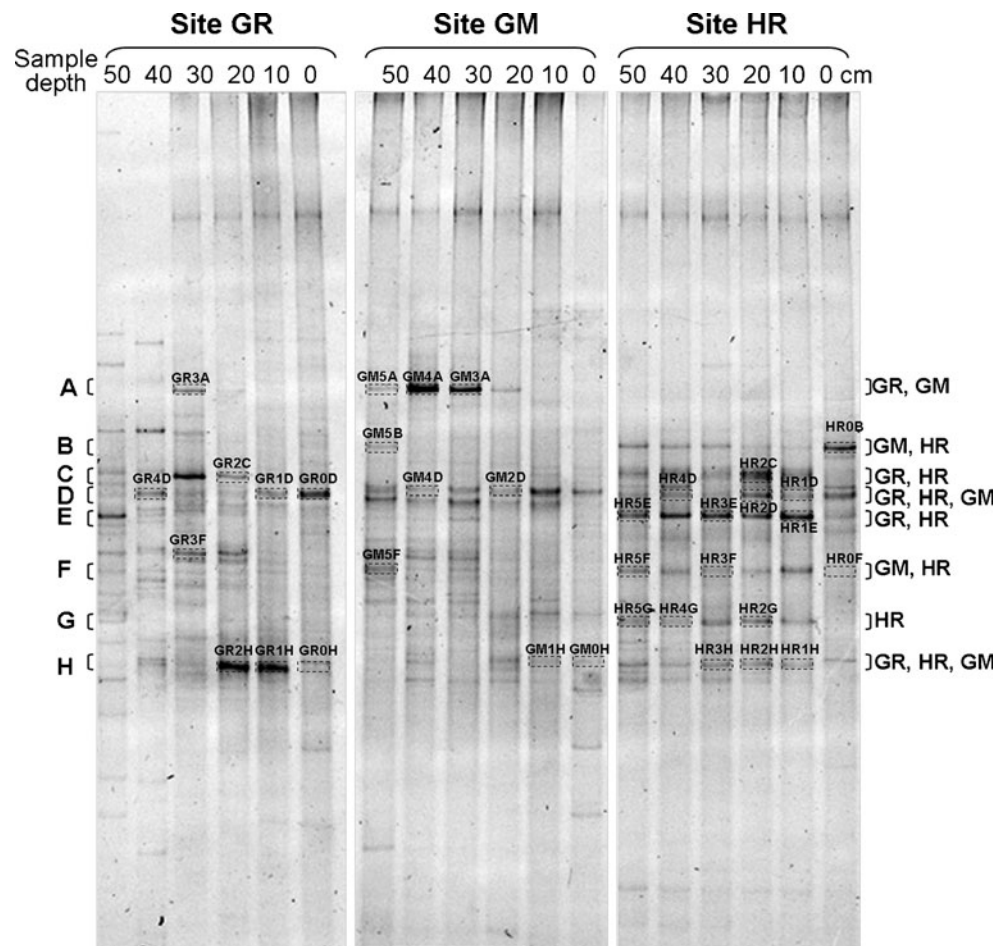
The band patterns of the DGGE profiles of the *dsrB* gene revealed that the composition of SRP varied between the sites in the Danshuei River estuary (Fig. 3). The DGGE profile patterns were well separated in two riverside localities, as well as mangrove and non-mangrove-vegetated habitats (Table 1). Eight major bands (labeled as A, B, C, D, E, F, G, and H) were chosen to express the distribution of the dominant groups of SRP. Position A was mainly distributed in the GR and GM. Positions B and F appeared at sites GM and HR. Positions C and E occurred at both the riverside sites GR and HR. Positions D and H

were recorded from all the three sites, whereas position G was only recognized at site HR. Although the band patterns did not significantly differ with depth as the three sites were pooled together (Table 1), the bands within each site seemed to exhibit variations of depth zonation (Fig. 3).

DGGE profiles were separated into three distinct clusters using cluster analysis (Fig. 4). With the exception of surface sample at site GR (labeled as GR00), the community compositions of *dsrB* gene were well classified by site. The first cluster included five communities from all the subsurface layers at site GR (10–50 cm, labeled as GR10, GR20, GR30, GR40, and GR50; Fig. 4), with similarity values varying from 0.55 to 0.89. Two sub-clusters of middle (GR10, GR20, and GR30) and deep (GR40, GR50) zones were further separated along the depth. The second cluster consisted of the entire *dsrB* gene communities from site HR, with two distinct communities and similarity values ranging from 0.67 to 1.00. Here, the compositions of *dsrB* gene showed little zonal variation in the depth below the surface, where the composition differed greatly from that in the surface layer (HR00, Fig. 4). The last cluster consisted of all the *dsrB* gene communities from the site GM as well as the surface layer of site GR, with similarity values ranging from 0.17 to 0.93. Five communities were vertically separated at site GM, and the most similar community was observed in the deep layers of 40–50 cm (GM40 and GM50).

The DGGE profile patterns mostly correlated with a combination of salinity, TOC content, and reduced sulfur concentration according to BIO-ENV analysis (matching correlation,  $r_s=0.56$ , Table 2). Furthermore, the DGGE profile patterns also exhibited high correlations with a combination of TOC content and salinity ( $r_s=0.52$ ) as well as single salinity factor ( $r_s=0.51$ ; Table 2). In addition,

**Figure 3** DGGE profiles of *dsrB* gene at site GR, GM, and HR. Letter labels on the left column refer to dominant bands, while those on the right column refer to sites where dominant bands were observed. On the DGGE profiles, the bands marked in rectangle with *dash line* were chosen for further sequencing. In summary, band D and H were found at all three sites. Band A was found at sites GR and GM; band B and F were found at sites GM and HR; band C and E were found at sites GR and HR; and band G was only found at site HR. Each of the six depths sampled is shown on top



the profile patterns were also affected by reduced sulfur concentration, although to a lesser extent ( $r_s=0.45$ ; Table 2).

#### Phylogenetic Affiliations of the Studied Partial Sequences of *dsrB* Gene

The phylogenetic tree of partial sequences of *dsrB* gene is shown in Fig. 5. Most of the sequences were affiliated to

**Table 1** Comparisons of SSRP communities as expressed in DGGE pattern profiles among riverside locality (GR vs. HR), occurrence of vegetation (GR vs. GM), sites, and depths (surface and every 10 up to 50 cm depth) using ANOSIM

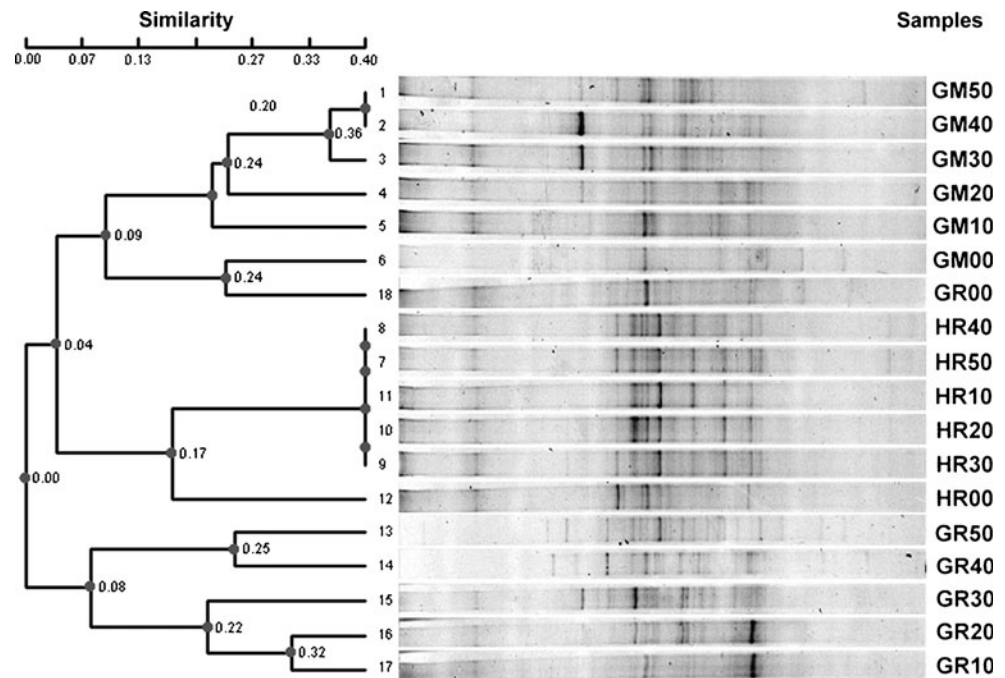
Factor	Global <i>R</i>	<i>p</i> Value	Significant pairwise test
Locality	0.87	0.002	
Vegetation	0.42	0.006	
Site	0.69	0.001	GM-GR (0.006), GM-HR (0.002), GR-HR (0.002)
Depth	-0.20	NS	

NS no significant difference at  $p<0.05$

those of  $\delta$ -Proteobacteria of sulfate reducers, while some sequences were related to those of the spore-forming sulfate reducers. One sequence, HR3F, resembled that of SiRP; however, no sequence showed affinity to that of SOP. Six clades with 15 gene phylotypes were identified according to the phylogenetic tree. The clade groups 1, 2, 4, 5, and 6 comprised more than two gene phylotypes, while only the clade group 3 had one phylotype (Fig. 5). In addition, sequences of groups 1 and 2 were found from all the three sites, whereas the sequence of group 3 was found at site GR only. In contrast, sequences of groups 4, 5, and 6 were primarily recorded from site HR, which comprised a freshwater environment.

Sequences of the first clade, group 1, were affiliated to the family Desulfobacteraceae. The first clade consisted of six phylotypes. The 1a phylotype, comprising GM5B sequence, was close to the *dsrB* sequence of *Desulfobacter fastidiosa* strain (AY268892, 87% similarity of protein sequences). The 1b phylotype, consisting of HR3E sequence, was closely related to the *dsrB* sequence of *Desulfatiferula olefinivorans* (DQ826725, 90% similarity). The 1c phylotype, possessing HR0F sequence, was closely related to the *dsrB* sequence of *Desulfosarcina variabilis* (AF191907, 77% similarity). The 1d phylotype, comprising

**Figure 4** Cluster analysis of *dsrB* gene based on DGGE band profiles across the three sites GR, GM, and HR using Ward's clustering algorithm with Pearson similarity coefficient (shown on furcating nodes and top scaling bar). The first two labels refer to sites and the last two numerals refer to depths



six sequences of GM2D, GR0H, GR1D, GR1H, GR2H, and GR4D, was similar to samples from Estuarine (FJ748824) and polluted harbor sediment (DQ112199), as well as landfill leachate aquifer (EF065086; 84–98%, 85–94%, and 84–99% similarity, respectively). The 1e phylotype, possessing four sequences of GM3A, GM4A, GM4D, and GM5A, was clustered with environmental clone from salt marsh (AY741561), coastal bay (AM408826), and anoxic basin sediments (AM236161; 90–93%, 91–92%, and 93–96% similarity, respectively). The 1f phylotype, consisting of only two sequences of GM0H and GM1H, was not found in both the identified strain and environmental clones in the current databases.

The third clade, group 3, was related to the family Desulfobulbaceae. The GR0D sequence was similar to the *dsrB* sequence of uncultured *Desulfobulbus* sp. (FJ544736, 81% similarity) and an environmental clone from landfill leachate aquifer (EF065035, 78% similarity).

The fourth clade, group 4, was related to the family Desulfovibrionaceae. Two phylotypes were observed in this clade (Fig. 5). The 4a phylotype, possessing HR0B sequence, was clustered with the *dsrB* sequence of *Desulfovibrio salexigens* (CP001349, 97% similarity) and *Desulfovibrio butyratiphilus* (AB490775, 63% similarity), as well as an environmental clone from sulfidogenic sludge biofilter (EU350982, 92% similarity). The 4b

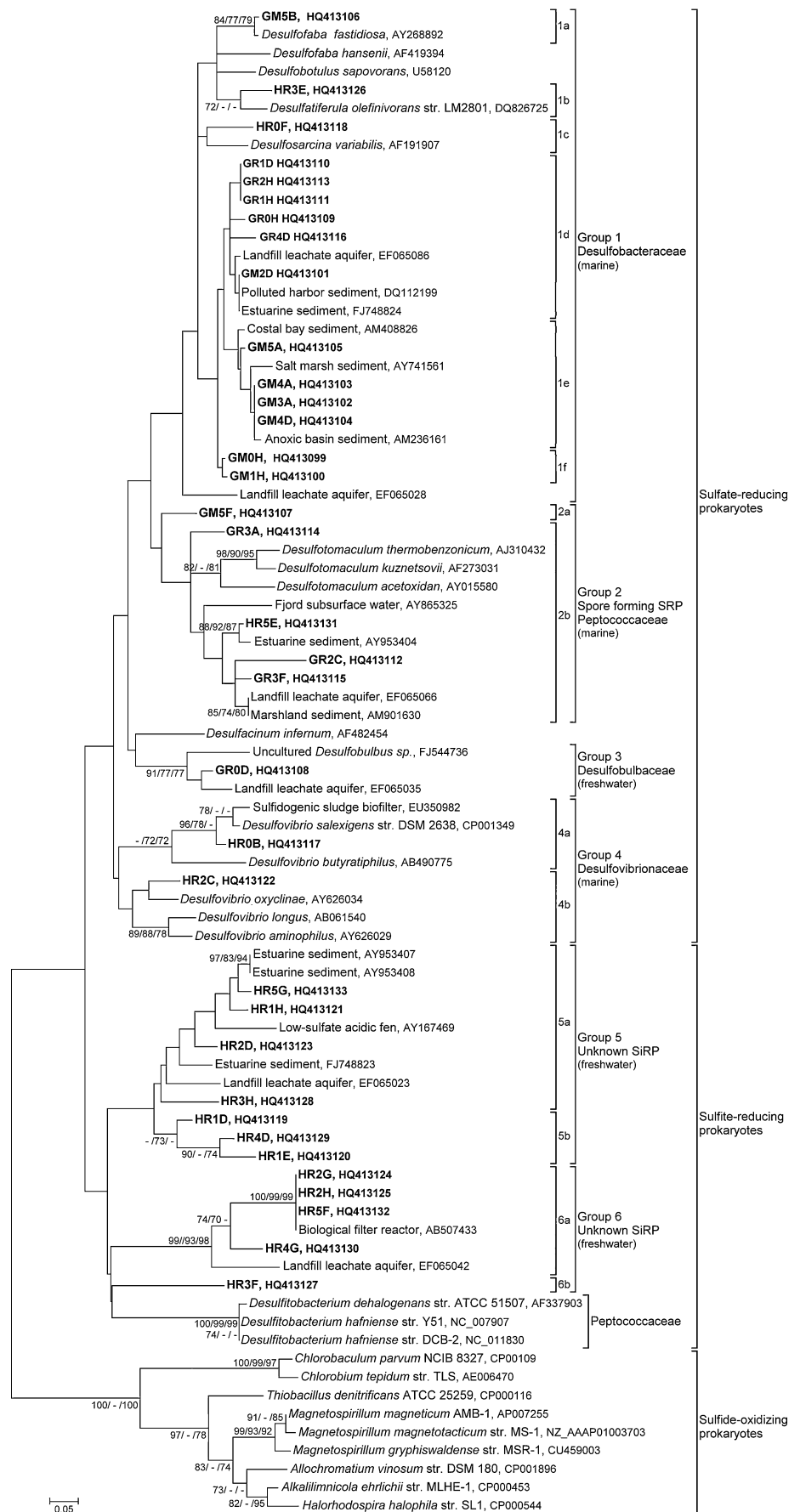
**Table 2** Relationships between SSRP communities as expressed in DGGE pattern profiles and six abiotic variables (salinity, and concentration of sulfate, sulfide, and reduced sulfur, as well as total organic carbon content and sampling depth) using BIO-ENV routine

No. of variables	$r_s$	Combination of abiotic variable
3	0.56	Salinity, TOC content, reduced sulfur concentration
2	0.52	Salinity, TOC content
1	0.51	Salinity
2	0.51	Salinity, reduced sulfur concentration
2	0.50	TOC, reduced sulfur concentration
4	0.48	Depth, salinity, TOC content, reduced sulfur concentration
4	0.47	Salinity, sulfate concentration, TOC content, reduced sulfur concentration
1	0.45	Reduced sulfur concentration
4	0.44	Salinity, sulfide concentration, TOC content, reduced sulfur concentration
5	0.43	Depth, salinity, sulfate concentration, TOC content, reduced sulfur concentration

Only the highest ten correlated relationships between SSRP communities and abiotic variables were shown here

$r_s$  Spearman rank correlation, TOC total organic carbon content

**Figure 5** Phylogenetic tree of *dsrB* gene based on DGGE bands using neighbor-joining method (NJ). Bootstrap values (>70%) of NJ as well as maximum parsimony and maximum likelihood methods are shown in the aforementioned order on the left of the relevant nodes. The first two letters refer to sites and the third numeral refers to depths. The last letter refers to major bands, A–H, which are shown in Fig. 3. SRP: sulfate-reducing prokaryotes, SiRP: sulfite-reducing prokaryotes





phylotype, consisting of HR2C sequence, was similar to the *dsrB* sequence of *Desulfovibrio oxycliniae* (AY626034), *Desulfovibrio longus* (AB061540), and *Desulfovibrio aminophilus* (AY626029; 86%, 80%, and 77% similarity, respectively).

The second clades, group 2, were related to the spore-forming SRP of the family Peptococcaceae. Two phylotypes were classified in this clade. The 2a phylotype, comprising GM5F, was neighbored to clusters associated with *dsrB* sequences of the genus *Desulfotomaculum*. The 2b phylotype, comprising GR2C, GR3A, GR3F, and HR5E sequences, was closely related to the *dsrB* sequences of *Desulfotomaculum thermobenzonicum* (AJ310432), *Desulfotomaculum kuznetsovii* (AF273031) and *Desulfotomaculum acetoxidans* (AY015580; 64–71%, 65–71%, and 68–78% similarity, respectively), the environmental clones from fjord subsurface water (78–82% similar to AY865325), landfill leachate aquifer (84–94% similarity to EF065066), marshland sediment (78–81% similarity to AM901630), and estuarine sediment (81–96% similarity to AY953404).

The fifth and sixth clades, groups 5 and 6, were mainly related to freshwater environmental sequences, and some of the members appeared to be affiliated to the identified SiRP of the family Peptococcaceae. Two phylotypes were observed in the fifth clade (Fig. 5). The 5a phylotype consisted of four sequences of HR1H, HR2D, HR3H, and HR5G, and was closely related to the environmental clones reported in freshwater-influenced estuarine sediments (76–96% similarity to AY953407, AY953408, and FJ748823), low-sulfate acidic fen (69–79% similarity to AY167469), and low-sulfate landfill leachate aquifer (75–79% similarity to EF065023). The 5b phylotype, possessing HR1D, HR1E, and HR4D sequences, was not found in either the identified strain or environmental clones in the current databases.

The sixth clade consisted of two phylotypes. The 6a phylotype, having four sequences of HR2G, HR2H, HR4G and HR5F, was similar to a partial sequence from biological filter reactor (82–100% similarity to AB507433) and low-sulfate landfill leachate aquifer (71% similarity to EF065042). Nevertheless, the 6b phylotype, with HR3F sequence, did not match with either the identified strain or environmental clones in the current databases.

## Discussion

In the estuary of the Danshuei River, SSRP communities varied between the lower estuarine brackish-water reach and the upper tidal freshwater reach and also differed between mangrove-vegetated and non-vegetated habitats. These discrepancies may be attributed to sulfate availability,

freshwater input, oxygen stress, and the presence or absence of mangrove vegetation.

## Effects of Salinity, Reduced Sulfur, and Organic Matters on SSRP Communities

The SSRP community structures in the Danshuei River are found to be highly associated with a combination of salinity, reduced sulfur concentration, and TOC content (Table 2), reflecting the effects of spatial gradient in salinity, deposition of reduced sulfur, and vegetation, respectively. When compared with these three factors, sulfate concentration appears to be less related to the SSRP community structures, because the correlation value decreased from 0.56 to 0.47 when the sulfate concentration was considered as an additional abiotic factor. Nevertheless, sulfate concentration may play a role indirectly in influencing the SSRP community distribution. Two processes, physical and biological, seem to be involved. First, the decrease in the sulfate level is a companion event with changing salinity along a marine-freshwater gradient. Second, this diminish is a metabolic consumption of sulfate by SRP [7, 8]. The sulfate concentration was positively correlated with water salinity, while negatively correlated with the deposition of sulfate-reducing products, such as reduced sulfur compound, AVS, and pyrite combined, particularly in a close system including deep sediments in lower estuarine area [7].

Previous studies have reported that organic matter is one of the important factors controlling SRP distribution [55]. Our results also show that the TOC content is greater at the mangrove-vegetated site GM than at non-vegetated site GR, suggesting that the level of organic matter derived from mangrove litter differs between these two sites, and in turn, is likely to be involved in the differentiation of SRP communities. In addition, certain SRP communities have been observed in habitats where fermentation is prevalent [39, 51]. Based on these data, we believe that the accumulation of organic matter can benefit growth of dissimilar SRP communities. Nevertheless, the pathway that becomes dominant depends on the levels of sulfate.

## SSRP Communities in the Tidal Freshwater Reach

Composition of SSRP communities, varying among the three study sites, was found to be highly related to the marine-freshwater gradient, reflecting positive correlations with the changes in the sulfate concentration and salinity. The importance of this gradient was also reported in studies on the distribution of denitrifiers in the same Danshuei River estuary [21] and the diversity of *dsrAB* gene sequences from mudflats in France [42].

SRP are normally distributed in sulfate-enriched but oxygen-depleted habitat, particularly in marine environment. Notably, our results show that both SRP and SiRP, for instance, members of groups 4, 5, and 6, occur in the tidal freshwater reach, which lack salinity and sulfate (Figs. 2 and 5). However, when examining sulfide concentration, it was higher in the tidal freshwater reach (site HR) than in the brackish-water reach (sites GR and GM). In addition, previous reports also showed that sulfate reduction was still rather active in this tidal freshwater reach (sulfate reducing rate at GR vs. HR—0.0654 vs. 13.1 mole  $\text{m}^{-2} \text{day}^{-1}$ ) [11]. We ruled out the possibility that sulfate is derived from salt intrusion with tides because no evidence showed the availability of sulfate at this site [62] (this study). Alternatively, leaking of some sulfurous compounds from a deeply buried ore or degradation and transformation from sulfur-containing pollutants may occur because the HR river bank has been a garbage dump site for decades [31]. Consequently, some unique SRP or SiRP strains may have been selected for this kind of low-sulfate and low-salinity setting. We found that band G series groups (HR2G, HR4G, and HR5G) detected from the HR area were phylogenetically affiliated to those recorded from low-sulfate acidic fen or landfill leachate aquifer of freshwater environment (Fig. 5 of this study) [55, 67]. In freshwater environments with limited sulfate supply, many *Desulfobulbus*, *Desulfovibrio*, and *Desulfomicrobium* species grow by fermenting pyruvate to form acetate, carbon dioxide, and hydrogen, in which acetogenic growth of SRP becomes dominant [6, 10, 39, 51].

#### Deposition of Reduced Sulfur Compounds and Vertical Distribution of SSRP Communities

On the basis of band patterns on DGGE profile, the SSRP communities in the brackish-water reach at Guandu exhibited more diverse compositions in deep than in shallow zones, and the delineation seemed to appear between 30- and 40-cm depth at site GR and between surface and 10 cm at site GM (Fig. 4). This discrepancy on the compositions between deep and shallow zones suggests that the SSRP in the present study sites are capable of utilizing different ecological setting, including saline and freshwater habitats, sulfate shortage, and deposition of reduced sulfur compound (Table 2) as shown in other studies [8, 26]. At the GR site, a sharp decrease in the pore-water sulfate and increase in reduced sulfur occurred at 20- and 40-cm depth, respectively (sulfate concentration at 20- vs. at 30-cm depth, 6.7 vs. 0.8 mM; reduced sulfur concentration at 30- vs. at 40-cm depth, 6.2 vs. 15.2  $\text{mg g}^{-1}$ ; Fig. 2). At the GM site, sulfate depletion also occurred at around 20-cm depth, whereas reduced sulfur compounds

began to accumulate 10 cm below the surface, a depth much shallower than that at GR. These data reflect that there exists a transitional zone, below which the sediments become steadily anoxic and reductive, thus favoring SRP growth because SRP is known to be obligate anaerobic prokaryotes [5]. In contrast, the SSRP band patterns at the HR site exhibited little depth variation. In particular, their compositions were rather similar through all depths below the 10-cm layer surface (Fig. 4). This vertically invariant compositional pattern may again be attributed to a constant anoxic condition but also to the scant occurrence of sulfate and reduced sulfur compounds through all depth layers.

#### Oxygen Stress on SSRP Communities in the Surface Sediments and Vegetated Habitat

The DGGE profiles of the *dsrB* gene at the three study sites revealed that the SSRP communities in the surface of 0–1 cm were less diverse than those in the deep layers of below 10-cm in depth (Fig. 4). This zonation pattern is likely to be related to oxygen stress. Oxygen in waterlog sediment penetrates only several millimeters of the top sediment [41], and the oxygenated depth is often deprived of SRP [7]. Nevertheless, certain species of SRP, such as *Desulfovibrio* and *Desulfobulbus* spp., can still survive in micro-aerobic condition or even tolerate high concentrations of oxygen [19, 35]. Based on these data, the SSRP communities found in the surface sediments in the present study may consist of oxygen-tolerable species that differ taxonomically from those dwelling in deep, anoxic environments.

At the site GM, the dissimilarity of the SRP assemblages increased as the depth decreased, and the SRP taxonomic richness (number of bands) became lower as they were distributed in shallower areas (Fig. 3). These trends suggest that the presence of vegetation may cause the disturbance of surface sediment, thus influencing the composition of the SRP communities. Holmer and Storkholm [26] proposed that macrophytes could affect sulfate reduction by altering the redox conditions of the sediments. Bioturbation, caused by emergent macrophytes or by various activities of macrobenthos, have been found to have negative effects on SRP distribution [41, 47]. It has been recorded that macroinfauna, such as polychaetes and crabs, are abundant in the Danshuei GM area, when compared with those in non-vegetated area [32]. The macrobenthos' burrowing, foraging, or irrigating activities can rework and oxygenate sediments up to a depth of 5–10 cm by polychaetes [54] and even deeper by crabs. We presume that oxygenation by mangrove roots and aeration by macroinfauna may act together to decrease the SRP richness at the GM site.

### Phylogenetic Relationships of *dsrB* Gene Sequences in the Tidal Freshwater Reach

The sequences of *dsrB* gene exhibited phylogenetically distinct SSRP communities among the three studied sites and the underlined processes were closely related to the marine-freshwater gradient (Fig. 5). The SSRP communities in the tidal freshwater habitat (HR) exhibited relatively high diversity. They were not only affiliated to prokaryotes living in freshwater habitats but also to those from marine environments. SSRP communities in the HR shared only one phylotype, 2b, with those at the other two sites in the lower reach, indicating the uniqueness of the *dsrB* genes in the HR. The phylotypes distributed in brackish lower estuarine GR and GM areas all showed affinity to marine SRP clades, whereas the phylotypes clustered to groups 5 and 6 were all from the freshwater habitat HR and resembled SiRP, particularly the phylotype 6b. Moreover, these freshwater-related phylotypes at HR were phylogenetically close to the genus *Desulfotobacterium*. Prokaryotes of this genus fail to use sulfate as an electron acceptor and are not active in typical marine environment [12, 64]. The other neighboring phylotypes in groups 5 and 6 were also reported from an environmental setting characterized as freshwater area of an estuary [42], low sulfate area in coastal zone [38, 67], low-sulfate acid fen [45], or biological filter reactor [68].

The marine-related phylotypes found at HR are affiliated to the genera *Desulfatiferula* (family Desulfobacteraceae), *Desulfosarcina*, *Desulfovibrio* (family Desulfovibrionaceae), and *Desulfotomaculum* (family Peptococcaceae). All the genera are capable to adapt to or tolerate low salinity or low sulfate supply [38, 42, 67]. In addition, they are found from a wide range of various environmental settings, such as from freshwater area of estuary [42] or coastal low sulfate area [38, 67]. Among them, *D. aminophilus*, which possesses the ability to break down amino acid anaerobically, was not only found in estuarine and marine environments, but also in freshwater area [3]. In a biofilter where the process of sulfate reduction was inhibited, *Desulfovibrio*-related fragments were also detected [59].

Moreover, the aforementioned prokaryotes and the others that the phylotypes from the site HR resembled are known to be capable of degrading pollutants [20, 31, 40]. For instance, the SiRP *Desulfotobacterium dehalogenans* and *Desulfotobacterium hafniense* are capable of performing dechlorination of chlorinated aromatic compounds [12, 64]. The SRP *D. thermobenzonicum* and *D. variabilis* can use benzoate or similar aromatic compounds as a carbon source [40, 62]. The SRP *D. olefinivorans* has the ability to scavenge aliphatic hydrocarbons of crude oil. In addition, some of these prokaryotes have been isolated from brackish sediment of a wastewater decantation facility of an oil refinery [17]. We believe that the sediments in the HR area may have been

exposed to anthropogenic pollution, because the adjacent river bank is used for garbage dumping and landfill [31] (the corresponding author's personal observation). Therefore, the group of SRP and SiRP that have ability to break down pollutants can colonize the HR area.

### Phylogenetic Relationships of *dsrB* Gene Sequences in the Brackish-water Reach

In the present study, the prokaryotes found in the brackish-water reach (GR and GM) all belong to SRP clades. When compared with those observed in GR and HR, the SRP phylotypes in the GM habitat were less diverse and dominated by group 1 sequences (i.e., 1a, 1d, 1e, and 1f, Fig. 5). Such homogeneous phylotype constituent may be attributed to a constant supply of sulfate and organic substrate (TOC content was the highest among the three sites, Fig. 2). Besides, one unique phylotype, group 3, was found at saline GR. Prokaryotes that exhibited close affinity to group 3 were reported from freshwater habitats, reflecting their physiological versatility [6, 39, 51].

Most of the phylotypes determined from the GR and GM sediments were affiliated to certain typical marine SRP such as the members of the genera *Desulfobaba* (family Desulfobacteraceae), *Desulfobotulus* (family Desulfobacteraceae), *Desulfatiferula* (family Desulfobacteraceae), *Desulfosarcina* (family Desulfobacteraceae), and *Desulfotomaculum* (family Peptococcaceae). The accumulated data demonstrate that these prokaryotes are detected from marine systems (Fig. 5), including salt marsh [4], lower estuary close to sea [34], coast with high sulfate [67], polluted harbor [69], and anoxic abyssal basin [43]. In addition, the *D. fastidiosa*-related strain was isolated from a sulfate-methane transition zone [1]. Some SRP, such as *Desulfovibrio* and *Desulfomicrobium* species, can oxidize lactate and ethanol to acetate when hydrogen is efficiently removed by hydrogen-consuming methanogens [6]. Sulfate reducers were also the dominant acetogenic prokaryotes in a methanogenic reactor that was used to treat whey [10]. In the present study from 40 to 50-cm depth at the GM site, we found a sequence, GM5B, which was closely related to *D. fastidiosa*. As sulfates have been very much depleted in this deep zone [11] (Fig. 2), we speculate that this strain may coexist with the methanogens that involve methanogenesis.

The phylotypes found at the GR site were phylogenetically affiliated to an unidentified clone of the genus *Desulfobulbus* (family Desulfobulbaceae; Fig. 5). *Desulfobulbus*-related prokaryotes appeared to grow by fermenting in the absence of an external electron acceptor, such as in the freshwater environment that lacks sulfate [42] or landfill leachate from polluted aquifer with low sulfate level (<1 mM) [67]. The GR reach is located in the lower

estuary of the Danshuei River and its salinity normally exceeds 22.0 [29]. However, the surface sediment at GR is occasionally or periodically subjected to freshwater [44], in which salinity could be lower than 10 (Fig. 2). Sulfate concentration lower than 2 mM has also been recorded in the deep layers in the vicinity of this site [21]. It is not uncommon that freshwater- and low-sulfate-associated *Desulfobulbus* SRP can occur in lower estuarine habitat with higher salinity because it has been reported that this strain is distributed in anoxic sediments of freshwater, brackish-water, and seawater [39]. As a result, such distribution pattern suggests that *Desulfobulbus*-related SRP found in GR surface sediments is capable of surviving in fluctuating salinity and sulfate concentrations.

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