

Genetic Diversity and Abundance of Flavobacterial Proteorhodopsin in China Seas^{∇†}

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Proteorhodopsin (PR) genes related to *Flavobacteria* were found to be highly diverse in the East and South China seas and displayed a distinct geographic pattern, which appeared to reflect cold versus warm adaptation when Global Oceanic Sampling database metagenomic data were included. Flavobacterial PR genes were more abundant offshore than nearshore, implying that inheritance of the PR gene could be important for *Flavobacteria* living in the oligotrophic environment.

Proteorhodopsin (PR) proteins are bacterial retinal binding membrane pigments that are predicted to have a phototrophic potential (4, 5, 27). Thousands of PR sequences have been retrieved from various marine (4–6, 9, 20–24, 31) and freshwater (3) environments. These PR genes belong to diverse groups of bacteria (4, 9, 13, 19, 21, 23, 27, 31), but are found mainly in three major marine phylum: *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes*. The diversity of PR genes related to *Proteobacteria* in nature has been studied extensively (21–23, 28). However, little is known about the distribution and diversity of PR genes related to *Bacteroidetes* in the marine environment.

Flavobacteria, an important class of *Bacteroidetes*, often make up a significant portion of marine microbial communities (1, 2, 14, 18). They are found both free-living and attached to organic aggregates and are considered as major mineralizers of organic matter (1, 7, 8, 18). A recent study showed that PR genes in *Flavobacteria* formed a unique cluster divergent from those in *Proteobacteria* and other taxa (19). So far, PR proteins from five different flavobacterial strains have been sequenced (see the primer design for reference), and the conservation of their PR genes allowed us to design a primer set specifically targeting this group of bacteria. Here, we report the diversity and geographic distribution of flavobacterial PR genes in the East and South China seas.

Surface water samples were collected using Niskin bottles from three stations (W1, W2 and W8) in the East China Sea on 27 to 28 November 2006 and two stations (A1 and A9) in the South China Sea on 19 to 20 January 2007 on board the R/V *Dong Fang Hong No. 2* (Fig. 1). Water samples from depths of 30 m, 50 m, and 70 m at station A1 and 60 m, 100 m, and 200 m at station W8 were also collected. Seawater was filtered onto a 0.22- μ m-pore-size polycarbonate filter (Millipore Co., Bedford, MA) after prefiltration through a 200- μ m-pore-size mesh, except for the surface samples from stations A1 and

A9, which were filtered onto a 3- μ m-pore-size polycarbonate filter (Millipore Co.) and subsequently onto a 0.22- μ m filter after the same prefiltration. Community DNA was extracted following a protocol described elsewhere (11). The primers used to amplify flavobacterial PR genes for both diversity and quantification studies were designed based on the PR sequences from five flavobacteria (see Fig. S1 in the supplemental material), including *Dokdonia donghaensis* MED134 (accession no. EAQ40507), *Polaribacter dokdonensis* MED152 (EAQ40925), *Polaribacter irgensii* 23-P (ZP_01117885), *Psychroflexus torquis* ATCC 700755 (ZP_01253360), and BAL38 (ZP_01734914). The forward primer (5'-GGCTATGATGGC WGCWT-3') and reverse primer (5'-CTTCACCAAWRTAW CCAGTWAC-3') flanked a 310-bp fragment, which contained several key amino acid positions for the functionality of PR, such as Asp97, Met/Leu/Gln105, and Glu108 (SAR86 eBAC31A08 numbering). PCRs were performed in a total volume of 50 μ l containing 5 to 10 ng of template DNA, 200

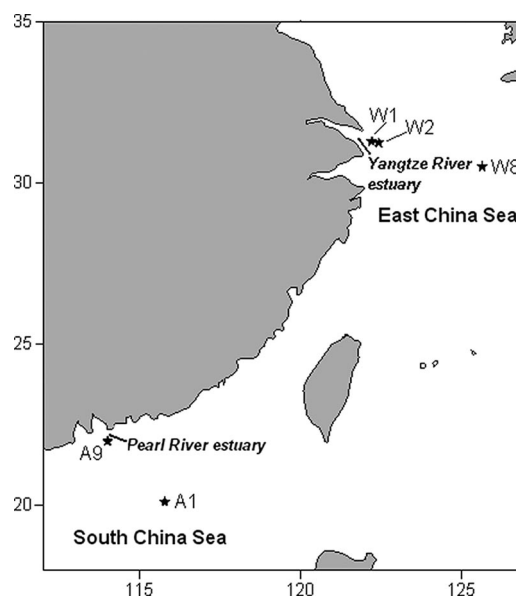
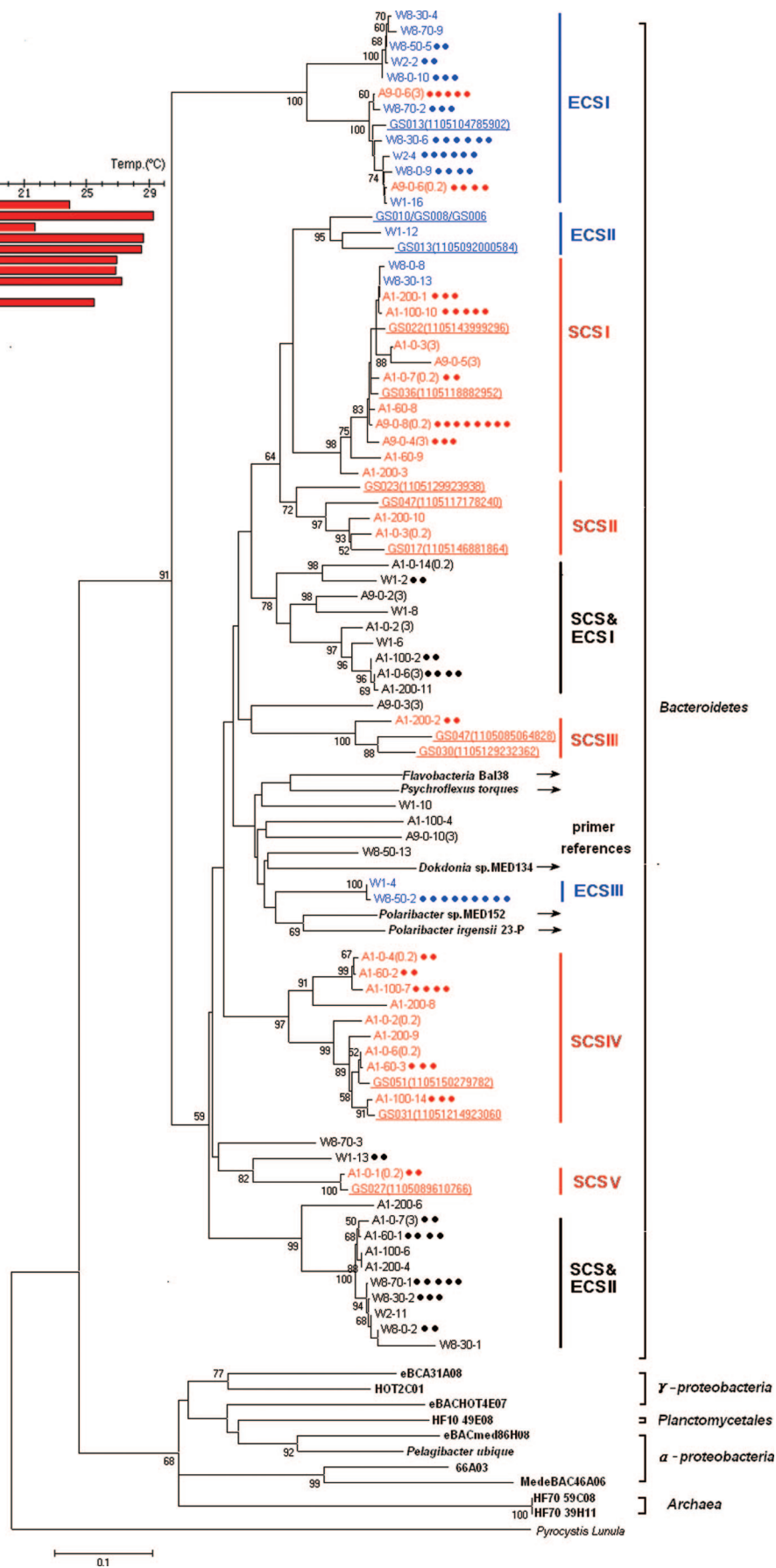
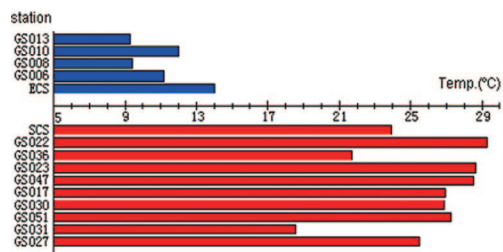


FIG. 1. Sampling locations (★) in the East and South China seas.

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μM deoxynucleoside triphosphates (dNTPs), 2.5 mM MgCl_2 , 0.5 μM of each primer, and 2.5 U of *ExTaq* polymerase (TaKaRa, Co., Dalian, China) under the following conditions: 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 54°C for 30 s, and 72°C for 1 min. PCR products of each sample were used to construct clone libraries, and about 10 positive clones were sequenced in each library. Multiple sequence alignments were performed using the CLUSTALX 1.81 program (30). The neighbor-joining (N-J) phylogenetic tree was inferred using Mega 4.0 (29). The rarefaction analysis and coverage estimation were conducted using DOTUR (25). The fraction of shared operational taxonomic units (OTU [at 5% genetic divergence]) between every two clone libraries was indicated by the classic Sørensen index (S) using the program SONS (26).

Real-time quantitative PCR (qPCR) was conducted with the ABI 7500 real-time PCR system (Applied Biosystems, CA) using the Sybr *Premix Ex Taq* kit (TaKaRa). The mixture solution (25 μl) and amplification conditions followed the manufacturer's protocol. Deionized water without DNA was used as a negative control. The standards were 10-fold serial dilutions of a pMD18-T plasmid (10^3 to 10^8 copies) containing a representative PR fragment from our clone library. The melting curve analysis was performed at the end of amplification.

Phylogenetic analysis. A total of 140 PR sequences were obtained from the East and South China seas. At 5% genetic divergence, the rarefaction curve of these PR sequences tended to reach a maximum (see Fig. S2 in the supplemental material), indicating that most flavobacterial PR genotypes in both China seas have been identified (the coverage by Chao1 richness estimation was 70%). Phylogenetic analysis (Fig. 2) showed that all of the PR sequences recovered from China seas were clustered with the five known flavobacterial PR sequences (nucleotide similarity of 65% to 100%) and distantly related to those from other phyla like *Proteobacteria* and *Archaea* (similarity of 48% to 64%), suggesting that our primers were suitable for amplifying the PR genes related to *Flavobacteria*. Our sequences could be divided into 25 OTU at 10% genetic divergence, indicating a high diversity of flavobacterial PR genes in the China seas. At an e value of 10^{-19} , a total of 83 different PR fragments were retrieved from the Global Ocean Sampling (GOS) metagenomic database using our sequences as queries for BLASTN search. The GOS hits came from a broad range of geographic sites, ranging from the North American coast through the Gulf of Mexico into the equatorial Pacific (20), suggesting a wide distribution of PR genes related to *Flavobacteria* in the global ocean.

Geographic pattern. Most of the flavobacterial PR genotypes detected in both China seas appeared to be region specific. For examples, five major SCS clusters (SCSI to

SCSV) were basically comprised of PR sequences from the South China Sea and three major ECS clusters (ECSI to ECSIII) were basically comprised of PR sequences from the East China Sea (Fig. 2). A few PR genotypes, such as those in clusters ECS & SCSII and ECS & SCSII (Fig. 2), were found in both China seas. The region-specific PR sequences accounted for about 2/3 of all the sequences recovered from each sea, respectively, reflecting a genetic divergence of PR between the two sea areas. Fifteen of 83 PR sequences retrieved from the GOS database were included in the phylogenetic analysis as they shared >90% similarity with our sequences. Interestingly, five GOS sequences from North Atlantic coastal water (GS006, -008, -010, and -013) were clustered with the PR sequences from the East China Sea in ECSI and ECSII (Fig. 2, clusters in blue) with sampling temperatures ranging from 9°C to 14°C (Fig. 2, left panel, bars in blue), while others (GS017, -022, -023, -027, -030, -031, -036, -047, and -051) from the tropical oceans were all clustered with the PR sequences from the South China Sea in SCSII to SCSV (clusters in red), with sampling temperatures ranging from 18°C to 29°C (bars in red). It is intriguing that common flavobacterial PR genotypes were found in both Pacific and Atlantic oceans where water temperatures are similar.

PR sequences from the five known flavobacteria were deeply branched within the *Bacteroidetes* group, probably due to their unique ecological adaptation. Strains Med134 and Med152 were isolated from the Mediterranean Sea (15), 23-P (16) and *Psychroflexus torquis* were isolated from Antarctica, and BAL38 was isolated from the Baltic Sea. We speculate that the two psychrophilic strains living in Antarctica might represent PR genotypes adapted to a lower temperature. It has been reported that temperature could affect the photocycle kinetics of PR (10). We hypothesize that temperature could be an important factor affecting the geographic distribution of different PR genotypes in the ocean. Further investigations are needed to fully understand the environmental impact on the global distribution of PR.

Free-living versus attached bacteria. Distinct PR genotypes were found between particle-attached (3- to 200- μm -size fraction) and free-living *Flavobacteria* (<0.22- μm -size fraction) at the surface water of station A1, as evidenced by the classic Sørensen index ($S = 0.2$) between the two clone libraries. However, no major difference ($S = 0.8$) between these two fractions was seen at station A9. This could be related to the rapid clogging on the 3- μm -filter as station A9 usually contains a large quantity of detritus from the Pearl River. As a result, all of the PR genotypes from the 0.22- μm filter could be found on the 3- μm filter at this station.

FIG. 2. Phylogenetic tree of flavobacterial PR genes from the East China Sea (ECS) and South China Sea (SCS) along with their homologs in the GOS database (underlined) relative to PR genes found in other major groups (bold). The five flavobacterial strains used for the primer design are marked by arrows. The N-J tree was constructed based on 310 nucleotide positions. Bootstrap values (1,000 resamplings) over 50% are indicated. The sequences obtained in this study are named by station (W1, W2, W8 in the ECS and A1 and A9 in the SCS), followed by the sampling depth (only for A1 and W8) and the clone number. Sequences postfixed with "(3)" or "(0.2)" represent PR from the 3- to 200- μm -size fraction or 0.22- to 3- μm -size fraction (only for A1 and A9 surface samples). Dots represent each clone with >95% DNA sequence identity to the representative sequence within one sample. Sampling temperatures in each GOS site and the two China seas (average temperature) are indicated in the left panel, and the sequences from the temperate and warm water are differentiated in blue and red. The omitted open reading frame accession no. of GS010/GS008/GS006 is (1105088900124)/(1105145485660)/(1105102499548).

Abundance of flavobacterial PR genes in the sea. The abundance of flavobacterial PR genes in the two China seas ranged from 54 to 4,354 copies/ml based on the qPCR estimation. The loss of DNA by the extraction procedure (DNA recovery rate of 25 to 50%) (11) was not counted in this estimation. The maximum value occurred at the surface water of station A1, the most oligotrophic site (chlorophyll *a*, 0.2 µg/liter) among the sampling areas, while the minimum value occurred at the 200-m depth of this station, the deepest of all of the samples. In the surface waters, flavobacterial PR genes at the offshore stations were more abundant than those at the nearshore stations. The quantity of PR genes increased from 1,822 copies/ml at a nearshore station (A9) to 4,354 copies/ml at an offshore station (A1) in the South China Sea and from ca. 900 copies/ml at nearshore stations (W1 and W2) to 2,136 copies/ml at an offshore station (W8) in the East China Sea. The nearshore stations in both seas were affected by a large amount of land-source organic matters and nutrients from either the Yangtze River (East China Sea) (32) or the Pearl River (South China Sea) (17) (chlorophyll *a*, 2.0 µg/liter at station W1 and 2.8 µg/liter at station A9). We speculate that the increase of PR abundance from inshore to offshore waters might be related to the trophic conditions. It has been demonstrated in the laboratory that a flavobacterium (MED134) with PR exhibited a light-stimulated growth under a carbon-limited condition (15). In the natural marine environment, it seems logical that a poor nutrient condition would favor more abundant PR genes to complement bacterial chemotrophic life style by phototrophy. In addition, certain PR types in *Alphaproteobacteria* were also found more abundant in the low-chlorophyll environments (6). Meanwhile, other studies suggested that the light benefit by PR in oligotrophic oceans might not be true for all individual taxa (12), with an explanation that PR might have other physiological role besides phototrophy.

The abundance of flavobacterial PR genes appeared to be low in the ocean. PR-containing *Flavobacteria* were roughly estimated to account for 0 to 1.6% of total bacteria in our sampling regions, similar to those reported in the North Atlantic Ocean (0 to 3%) using the same qPCR method but differently designed primers (6). The qPCR method applied here has been well calibrated. The standard curve for the quantification was linear ($R^2 > 0.999$) over a 6-log dynamic range with a PCR efficiency close to 100%. It is possible that the PCR primers may not cover all of the PR genotypes in *Flavobacteria*. However, given that highly diverse PR sequences were retrieved using this primer set, the quantification of flavobacterial PR genes in the China seas is probably close to the actual abundance.

This study provides a detailed analysis of the diversity and distribution of PR genes related to *Flavobacteria* in the natural marine environment. A temperature-related geographic pattern was noticed in this PR type. Whether such a pattern exists in other bacterial PR proteins is still not known and deserves further investigation.

Nucleotide sequence accession numbers. The PR sequences obtained in this study have been deposited in GenBank under accession no. EU683472 to EU683610.

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