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Genetic diversity of aerobic anoxygenic photosynthetic bacteria in open ocean surface waters and upper twilight zones

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Abstract Aerobic anoxygenic phototrophic bacteria (AAPB) represent a widespread mixotrophic bacterial group in marine ecosystems. Here we investigated AAPB genetic diversity in the surface waters and upper twilight zones of the central Pacific, Atlantic, and Indian oceans by amplifying an AAPB marker gene (*pufM*, encoding photosynthetic reaction center small subunit) directly from bacterioplankton community DNA. Phylogenetic and statistical analysis of 267 *pufM* partial sequences in six clone libraries revealed a high diversity pattern in open ocean AAPB communities. Various AAPB subgroups belonging to *Alpha-* and *Gamma-proteobacteria* were found in both surface and upper twilight zone waters. In most samples, subgroups in which no pure culture was isolated as yet were predominant. By sampling a wide size range of bacterioplankton (0.22–200 μm) and introducing nested PCR to amplification, we retrieved abundant *pufM* fragments (136 sequences in 37 OTUs) directly from upper twilight zone samples. AAPB populations in upper twilight zones covered major subgroups found in surface waters and had a slightly lower diversity, higher dominance, and lower GC and GC_3 contents in *pufM* genes than those in surface AAPB populations. These diversity data combined with previous BChl.*a* data in upper twilight zones support the hypothesis that AAPB may be present below euphotic zones based on the speculation that AAPB can utilize the dim light in twilight zones as a supplement to energy supply in their heterotrophic lives.

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Introduction

Aerobic anoxygenic phototrophic bacteria (AAPB) contain bacteriochlorophyll *a* (BChl.*a*) and represent an important functional bacterial group in marine ecosystems (Kolber et al. 2000, 2001). As heterotrophic bacteria, AAPB are actively involved in marine microbial loops and contribute to the transformation of dissolved organic matter; meanwhile, with BChl.*a*-based photosynthesis as a complement to their heterotrophic life (Kolber et al. 2001; Beatty 2002; Jiao et al. 2007), AAPB can use light for generation of ATP and thus reduce the consumption of organic carbon, contributing indirectly to carbon fixation by the ocean (Jiao et al. 2006). The mixotrophic lifestyle and widespread occurrence of these photoheterotrophs in marine ecosystems are challenging our views of the carbon and energy budgets in the oceans (Eiler 2006).

Since the first discovery of marine AAPB isolates at the end of the 1970s in the Bay of Tokyo (Shiba et al. 1979) and the first confirmation of their high abundance in the ocean in the early 2000s (Kolber et al. 2000, 2001), our understanding of marine AAPB abundance and diversity, and their ecological functions has been greatly extended. AAPB abundance has been investigated in various marine regimes by detecting BChl.*a* fluorescence signals, including the northeast Pacific (11.3 ± 1.7%; Kolber et al. 2001), the Baltic sea (1–12% of total prokaryotes; Masin et al. 2006), Mid-Atlantic Bight (5–16%; Cottrell et al. 2006), central North Pacific Gyre (5% or less; Cottrell et al. 2006), the northwest Atlantic (1–9%; Sieracki et al. 2006),

the Chinese marginal seas (0.5–11.6%; Zhang and Jiao 2007), the Delaware and Chesapeake estuaries (2.5 ± 0.3 to 14 ± 0.9%; Waidner and Kirchman 2007), and South Pacific Ocean (0.1–24.2%; Lami et al. 2007). Recently, Jiao et al. (2007) performed a comprehensive survey of global surface oceans and obtained a continuous distribution pattern of AAPB in the Pacific, Atlantic, and Indian Oceans for the first time. The stations investigated in these reports covered all major marine regimes except both polar regions (latitude > 75°N/S). In addition to saline systems, AAPB were also found to be widely distributed in freshwaters (e.g., lake, reservoir, and fish pond) with the fraction ranging from less than 1–21% in prokaryote communities (Masín et al. 2008). Their wide distribution in global aquatic ecosystems reinforces the significance of AAPB with respect to their roles in aquatic food webs and elemental biogeochemical cycles, evolutionary history (Beatty 2002), and co-evolution with environments (Jiao et al. 2007; Zeng and Jiao 2007).

In contrast to the abundant data on AAPB's distribution in various natural environments, few AAPB pure cultures have been isolated to date. The cultured AAPB species are mainly limited to members of *Alpha-proteobacteria* (dominated by the two genera *Roseobacter* and *Erythrobacter*), *Beta-proteobacteria* (only freshwater species), and *Gamma-proteobacteria* (only one marine species identified as yet; Fuchs et al. 2007) (For reviews, see Yurkov and Beatty 1998; Beatty 2002; Rathgeber et al. 2004). To fill the gap between the ecologically wide distribution and the few cultures available, culture-independent molecular methods were introduced to assess AAPB diversity in natural environments. *PufL/M* genes coding for large and small subunits of photosynthetic reaction center complex were first used as an indicator for surveys of AAPB genetic diversity in the ocean by Beja et al. (2002). An unexpected high AAPB diversity was revealed. This finding was confirmed by further surveys in the Red and Mediterranean Seas (Oz et al. 2005), California coast (Schwalbach and Fuhrman 2005), Chinese marginal seas (Du et al. 2006; Hu et al. 2006), the Pacific Ocean (Hu et al. 2006), Baltic Sea (Salka et al. 2008), and the Delaware estuary (Waidner and Kirchman 2008) and by two comprehensive investigations in global oceans (Jiao et al. 2007; Yutin et al. 2007). Unfortunately, current diversity data are limited to marine surface waters and little is known about AAPB community composition in deep waters, especially in the twilight zone, a dimly lit area 100–1,000 m below the surface, which comprises a large part of ocean depth and is a critical link between the surface and the deep ocean.

To explore AAPB diversity under ocean surface and to improve our understanding of AAPB biogeography in global oceans, we sampled seawater from the surface and upper twilight zones in the Pacific, Atlantic, and Indian Oceans during 2005 and made a systematic comparison of AAPB community structures in surface and below the euphotic zone. Additionally, statistical analysis of diversity data was performed to provide more insight into the mechanism of AAPB's adaptation to vertical ocean environments and their co-evolution with surrounding environments.

Materials and methods

Sampling sites and environmental variables

Surface (0 m) and upper twilight zone (200 m) waters were sampled from the three oceans during 2005: the Pacific Ocean on April 10, the Atlantic Ocean on October 17, and the Indian Ocean on December 26. This sampling was a part of marine scientific surveys during a global cruise (China R/V Ocean No.1) as a memorial of the 600-year anniversary of Admiral Zheng He's expedition voyages (1405–1433) to the occident. Sampling was not carried out in the same month due to limitations in shipping time. However, open ocean is a relatively stable system with respect to the physical-chemical conditions at the seasonal or annual scale and therefore the design served our purpose of addressing AAPB biogeography and their composition in deep seawaters.

All environmental variables were derived from the level-3 standard mapped images in the NASA MODIS ocean color datasets (http://oceancolor.gsfc.nasa.gov/cgi/level3.pl). Euphotic zone depth, surface temperature, surface particulate organic carbon (POC) concentration, and chlorophyll *a* (Chl.*a*) concentration were derived from the monthly mean Aqua-MODIS Euphotic Depth [Lee] dataset, the monthly mean Aqua-MODIS SST [11 μ day] dataset, the monthly mean Aqua-MODIS POC dataset, and the monthly mean Aqua-MODIS chlorophyll dataset, respectively, in corresponding months. Euphotic zone depth and surface POC data in 2005 were not available in MODIS datasets and we referred to those in the corresponding months of 2007, since the inter-annual variation of open ocean physical–chemical conditions is not significant. Detailed sampling information and environmental variables are summarized in Table 1.

Samples collection and community DNA extraction

Water samples (1–3 L) were pre-filtered through a 200 μm mesh and subsequently filtered onto 0.22 μm pore size filters (Pall, Gelman Sciences Inc). Filters were immediately frozen in liquid nitrogen and then transferred to −80°C for storage. Microbial community DNA was extracted using the hot SDS, phenol/chloroform/isoamyl alcohol, ethanol precipitation extraction protocol initially

Table 1 Sampling locations and environmental variables

Station	Longitude	Latitude	Sampling time	Sampling layer	Chl.*a* (mg/m^3)	Euphotic zone depth (m)[a]	Surface temp. (°C)	Surface POC (mg/m^3)[a]	Light intensity at 200 m (% surface PAR)[b]
PO-05Apr	149.38 W	16.39 N	Apr. 10, 2005	0 m, 200 m	0.04	120	23.5	24.5	0.046
AO-05Oct	45.06 W	14.83 N	Oct. 17, 2005	0 m, 200 m	0.09	115	27.6	28.2	0.033
IO-05Dec	75.85 E	10.01 S	Dec. 26, 2005	0 m, 200 m	0.15	90	28.8	33.9	0.004

Surface chlorophyll *a* (Chl.*a*) concentrations, euphotic zone depth, surface temperature, and surface POC were derived from the monthly mean level-3 standard mapped images (Aqua-MODIS) in corresponding months in the NASA MODIS ocean color datasets

POC particulate organic carbon, *PO* the Pacific Ocean, *AO* the Atlantic Ocean, *IO* the Indian Ocean

[a] Refer to MODIS data in the corresponding months of 2007

[b] Calculated based on the MODIS attenuation coefficient data in percentage of surface photosynthetic available radiation (PAR)

described by Fuhrman et al. (1988) with minor modifications by Zeng et al. (2004). DNA size and quality were determined using 1% agarose gel electrophoresis and the absorption at 260 nm obtained with a SmartSpec plus spectrophotometer (Bio-Rad Laboratories Inc., CA, USA).

Target gene and primers

AAPB is a functional group with phylogenetically different species covering *Alpha*-3 and *Alpha*-4 *proteobacteria*, *Beta-proteobacteria* and *Gamma-proteobacteria* (Yurkov and Beatty 1998; Rathgeber et al. 2004; Fuchs et al. 2007). In the two major marine AAPB genera, *Roseobacter* and *Erythrobacter*, both AAPB and non-AAPB species occur, and therefore design of AAPB specific 16S rRNA gene primers is practically impossible. A marker gene for aerobic anoxygenic photosynthesis, *pufM*, which encodes the photosynthetic reaction centre small subunit, has been widely used to investigate AAPB diversity in marine environments (Beja et al. 2002; Oz et al. 2005; Yutin et al. 2005; Du et al. 2006; Hu et al. 2006; Jiao et al. 2007; Salka et al. 2008; Waidner and Kirchman 2008). Various primers have been designed for amplifying full-length or partial *pufLM* gene fragments (Achenbach et al. 2001; Beja et al. 2002; Yutin et al. 2005; Waidner and Kirchman 2008). To make our results comparable with previous data, we chose the primer set identical to that previously used in Achenbach et al. 2001, Beja et al. 2002, Du et al. 2006, Hu et al. 2006, and Jiao et al. 2007: forward *pufM* primer 557: 5′-TACGGSAACCTGTWCTAC-3′, and reverse *pufM* primer 750: 5′-CCATSGTCCAGCGCCAGAA-3′. For deep layer samples which contained very low AAPB biomass, *pufLM* genes were preamplified with *pufL* forward primer (5′-CTKTTCGACTTCTGGGTSGG-3′; Beja et al. 2002) and reverse *pufM* primer 750 prior to a second-round *pufM* amplification. All oligonucleotide primers were synthesized by a commercial company (Sangon Co., Shanghai, China).

PCR amplification and clone library analysis

Partial *pufM* gene fragments were amplified using PCR with 50 μL reaction solution containing 5 μL of 10× PCR buffer with 25 mM $MgCl_2$, 0.5 μL of each deoxynucleoside triphosphate (dNTP) at 20 mM, 0.5 μL of each forward and reverse primer at 40 mM, 0.5 U of *Taq* DNA polymerase (TaKaRa Co., Dalian, China), and 2 μL DNA templates with a final mass of 20–25 ng. Reactions were cycled on a T3 thermocycler (Biometra Co., Germany) with the following program: 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min with a final extension step at 72°C for 5 min. For nested PCR, the first-round amplification used the same parameters as above except for the extension time (2 min used) and total cycles (15 here). One milliliter of amplification products were used as templates for the second-round amplification. Three independent PCR products were mixed and gel-purified using a Gel Extraction Kit (Sangon Co., Shanghai, China) according to the manufacturer's instructions. Ligation into pMD18-T vector (TaKaRa Co., Dalian, China) and transformation into *Escherichia coli* DH5a (TaKaRa Co., Dalian, China) were performed following standard procedures. The ampicillin-resistant clones were randomly picked out and screened for target inserts using colony PCR with sequencing primers RV-M (5′-AGCGGATAACAA TTTCACACAGG-3′) and M13-47 (5′-CGCCAGGGT TTTCCCAGTCACGAC-3′). Six clone libraries were constructed and more than 300 recombinant DNA clones (50 for each library) were screened.

Sequencing and phylogenetic analysis

Fifty positive clones in each library were subjected to the extraction of recombinant plasmid DNA and subsequent sequencing on an ABI 377A automated sequencer (Applied Biosystems) with M13-47 as sequencing primer. To detect chimera sequence, the nucleotide positions

1–96 and 97–192 of each sequence obtained were separately subjected to phylogenetic analysis. Multiple alignments of both regions were performed using the program MEGA 4.0 (Tamura et al. 2007) and edited manually. Two neighbor-joining trees, $Tree_{1-96}$ and $Tree_{97-192}$, were constructed with Jukes-Cantor correction and default parameters. Sequences that occupied different positions in both trees (i.e., distributed in different phylogenetic subgroups) were identified as chimeras and omitted from further analysis.

All those identified as normal sequences and reference sequences were aligned as described above. To obtain a compact tree, *pufM* sequences from the same station were combined into distinct groups prior to phylogenetic tree inference based on 6% nucleotide sequence difference, a cutoff value which was proposed for discriminating anoxygenic phototrophic species based on *pufM* sequences (Zeng et al. 2007). One sequence from each group combined with reference sequences from cultures and environmental clones were subjected to neighbor-joining tree inference with MEGA 4.0 (Tamura et al. 2007). Bootstrap values were obtained with 1,000 resamplings. The *pufM* sequence of *Chloroflexus aurantiacus* was used as the outgroup. The phylogenetic tree was further manually optimized by deleting the sequences or clades that were phylogenetically distantly related to our sequences.

To determine if these libraries are significantly different from one another, ∫-LIBSHUFF analysis was performed (http://schloss.micro.umass.edu/software/slibshuff; Schloss et al. 2004). Similarity of these sequences with those from other environments was determined by blasting representative sequences of each phylogenetic subgroup with GenBank records (http://www.ncbi.nlm.nih.gov/BLAST/) and marine microbial community metagenome datasets (CAMERA online database, http://camera.calit2.net/), including the metagenomes in Hawaii ocean time-series (HOT) depths (DeLong et al. 2006), Sargasso sea surface water (Venter et al. 2004), deep Mediterranean bathypelagic habitat (DEEPMED) (Martín-Cuadrado et al. 2007), and the global ocean sampling (GOS) expedition (Rusch et al. 2007).

Statistical analysis

The diversity of genotypes represented in *pufM* gene libraries was analyzed using rarefaction analysis with the program DOTUR (Schloss and Handelsman 2005). Rarefaction could determine if a sufficient number of clones were screened to estimate total diversity in each clone library and also could compare the relative richness among different communities. For each library, various statistical parameters were presented: *Taxa*, number of distinct OTUs in each library based on 6% nucleotide difference; *Individuals*, number of total positive clones screened out in each library; *Coverage*, derived from the equation $Coverage = 1 - (N/Individuals)$, where N is the number of clones that occurred only once; Diversity indices (*Dominance* and *Shannon*), calculated using the statistical program PAST (http://folk.uio.no/ohammer/past). *Shannon* index was a measure of the structural diversity of the microbial community and was calculated from the number and relative abundance of OTUs in the libraries (Odum 1971). *Dominance* describes the extent of dominance by individual OTUs (Odum 1971). *Distinct sequence*, number of distinct sequences in each library that had at least one nucleotide difference; *GC content*, G + C percentage in the base composition of all partial *pufM* sequences in each library; GC_3 *content*, G + C percentage in the base composition of all the third codons; *Maximum sequence distance*, the maximum distance among *pufM* sequences in each library, calculated with the program DNADIST program of the PHYLIP 3.63 package (http://evolution.genetics.washington.edu/phylip.html); and *Mean sequence distance*, average of all distance data for each library.

Sequence accession numbers

All partial *pufM* gene sequences reported in this study were deposited in GenBank under the accession numbers EU862408–EU862477.

Results

Sampling and environmental variables

Sampling stations were located in the central equatorial regions of the Pacific, Atlantic, and Indian Oceans, representing typical oligotrophic oceanic environments as seen from Chl.*a* data (Table 1). The Chl.*a* concentration in the station IO-05Dec was slightly higher than the other two stations. Surface temperature and particulate organic carbon (POC) showed a similar trend to Chl.*a* with all high values occurring at the Indian Ocean station.

The upper twilight zones were sampled at 200 m. As seen from the MODIS euphotic zone depth data in 2007, euphotic zones were limited to the upper 120 m. In optimal conditions, the euphotic zone in the open ocean may reach ca. 180 m at most. Thus the 200 m depth sampled in this study exactly represented the upper twilight zones where light intensity was extremely low ranging from 0.004 to 0.044% of sea surface photosynthetic available radiation (PAR) (Table 1). We tried greater depths (500 m and even below) for amplifying *pufM* but did not obtained any amplification product.

Table 2 Statistical analysis of *pufM* clones in each library and phylogenetic subgroup

Subgroup	Station						Total
	PO-0 m	PO-200 m	AO-0 m	AO-200 m	IO-0 m	IO-200 m	
Unknown Group I	31	16					47
Unknown Group II	7		9				16
Unknown Group III					24	11	35
Unknown Group IV			14				14
Unknown Group V					2	1	3
Unknown Group VI			9		6	11	26
Unknown Group VII				38		5	43
Gamma-like Group I		7					7
Gamma-like Group II		8					8
Gamma-like Group III			1	4			5
Gamma-like Group IV					5	13	18
Erythrobacter-like Group I	3		6		1		10
Sphingomonas-like Group I					1	1	2
Roseobacter-like Group I			5				5
Roseobacter-like Group II				3			3
Roseobacter-like Group III		9		2		5	16
Roseobacter-like Group IV					3	2	5
Roseobacter-like Group V	4						4
Total	45 (2/3)	40 (4/6)	44 (1/5)	47 (2/1)	42 (3/5)	49 (0/1)	267 (12/21)

Subgroups were defined based on the phylogenetic analysis of partial *pufM* sequences (see "Materials and methods"). Listed in parentheses are numbers of negative clones and chimera sequences that were screened out from 50 clones examined in each clone library

PufM clone library analysis

Totally, 300 *pufM* clones in the six libraries were screened with 50 for each library. Phylogenetic analysis showed that 3/6, 5/1, and 5/1 chimera sequences occurred in the libraries PO-0 m/200 m, AO-0 m/200 m, and IO-0 m/200 m, respectively ($Tree_{1–96}$ and $Tree_{97–192}$ not shown). Finally, 267 normal sequences were obtained and chimeras accounted for 7.0% of total sequences (Table 2), slightly lower than the frequency proposed for 16S rRNA gene clone libraries (average 9.0%, Ashelford et al. 2006), suggesting that functional gene diversity surveys should be carried out with caution against contamination by chimeras. For sampling in each clone library, all *Coverage* values were higher than 90% (Table 3), indicating that the clone numbers screened in each library were statistically sufficient for assessment of *pufM* gene diversity.

Sequence statistical analysis

Within the 267 normal sequences, identical sequences accounted for 73.8% (197/267). Seventy distinct sequences with at least one nucleotide difference were finally identified (9–15 in each library). Interestingly, no identical sequences were found between any two samples, suggesting each station was inhabited by different AAPB species. In accordance with the above *Coverage* data, the nearly saturated rarefaction curves on the 1% (Fig. 1a) and 6% (Fig. 1b) difference levels revealed that AAPB diversity was adequately sampled.

To compare the diversity between different libraries, *Shannon* and *Dominance* indices were measured (Table 3). Generally, surface stations showed higher *Shannon* values than deep sea stations, suggesting that surface oceans may be inhabited by more diverse AAPB. *Dominance* did not show a similar trend to *Shannon*. High *Dominance* indicated that a small portion of species contributed a large portion of clones in the libraries. In the Pacific and Indian Oceans, the surface stations showed higher *Dominance* than the deep stations; whereas for the Atlantic Ocean, high *Dominance* occurred at the deep station. The highest value appeared at the station AO-200 m, indicating that the AO-200 m library was dominated by very few species. In contrast to diversity indices, the maximum and mean sequence distance among *pufM* sequences showed no significant variation between surface and deep stations (Table 4). The results suggest that AAPB photosynthetic genes may undergo the same degree of evolution in both surface and deep oceans irrespective of the differences in light and nutrient supply. GC content in genomes or genomic fragments is another indicator of species adaptation to environments (Foerstner et al. 2005). Based on our data concerning the GC content of all *pufM* nucleotide positions and of the third codon positions (Table 3), surface ocean

Table 3 Statistical analysis of *pufM* sequences in each station

	PO-0 m	PO-200 m	AO-0 m	AO-200 m	IO-0 m	IO-200 m
Taxa[a]	8	7	10	4	8	7
Individuals	45	40	44	47	42	49
Coverage (%)	97.8	97.5	93.2	100	95.2	95.9
Dominance	0.18	0.3481	0.1364	0.6668	0.1945	0.3696
Shannon	1.857	1.433	2.096	0.6915	1.778	1.363
Distinct sequence[b]	9	13	12	9	12	15
GC content (%)	61.7	59.0	63.1	59.4	61.4	60.8
GC_3 content[c] (%)	60.5	58.3	57.1	56.9	59.1	57.8
Maximum sequence distance	0.2905	0.2924	0.3666	0.3220	0.3130	0.3146
Mean sequence distance	0.1615	0.1808	0.2472	0.2072	0.2048	0.1832

Coverage was derived from the equation *Coverage* = 1 − (*N*/*Individuals*), where *N* is the number of clones that occurred only once. *Maximum sequence distance* represents the maximum distance among *pufM* sequences in each library

[a] Based on 6% nucleotide difference

[b] Based on 1% nucleotide difference

[c] GC content on the third codon position

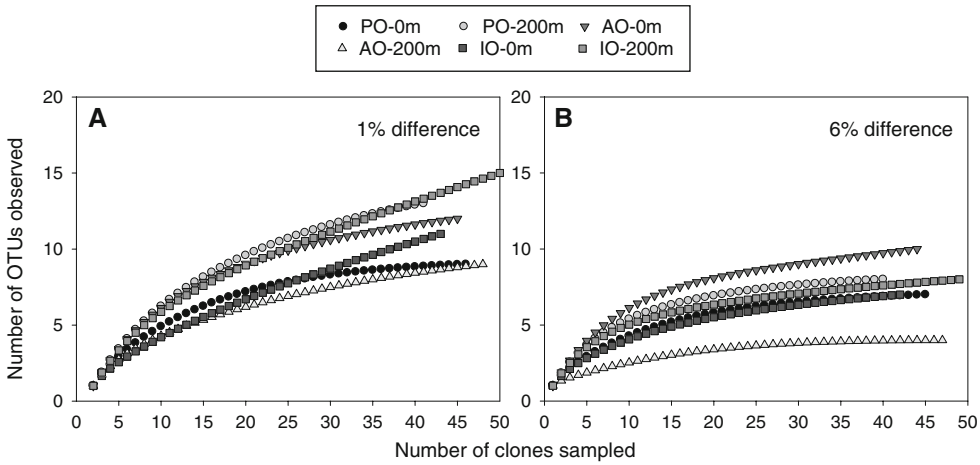

Fig. 1 Rarefaction analysis of six *pufM* clone libraries on 1% (**a**) and 6% (**b**) nucleotide sequence difference

fragments showed slightly higher GC and GC_3 contents (average 61.1 and 58.9%) than deep ocean fragments (average 59.7 and 57.7%). The same trend appeared in all three ocean regions investigated.

AAPB diversity pattern

Phylogenetic analysis grouped *pufM* sequences into 18 subgroups (Fig. 2), including five *Roseobacter*-like, one *Erythrobacter*-like, one *Sphingomonas*-like, four *Gamma-proteobacteria*-like subgroups, and seven not yet identified subgroups (Table 2, Fig. 2). AAPB belonging to *Beta-proteobacteria* were not found. Sequences within unknown subgroups account for 71.5% of total clones (Fig. 3), suggesting an abundant unknown diversity in open oceans and also the insufficiency of current efforts on isolating AAPB from oligotrophic ocean environments. *Roseobacter*-like and *Gamma-proteobacteria* -like subgroups were the secondly most abundant groups (12.4 and 11.6% of total clones, respectively) (Fig. 3). Each library was dominated by one of the *Unknown* subgroups except IO-200 m where *Gamma-proteobacteria*-like group IV dominated (27%).

∫-Libshuff analysis showed no significant difference between surface and upper twilight AAPB populations and neither among different oceans (Table 4). This indicated that AAPB populations in open oceans tend to be homogeneous irrespectively of locations and layers. As for the six libraries, significant difference (both P_{XY} and $P_{YX} < 0.05$) existed between AO-0 m and IO-0 m/PO-0 m and between PO-200 m and AO-200 m/IO-200 m/PO-200 m (Table 4), accounting for 1/3 of total *P*-value data (5 significant P_{XY} or P_{YX} in total 15 P_{XY} or P_{YX} values). This result indicated that no significant difference was observed between most of clone libraries.

Table 4 *P*-values in ∫-Libshuff analysis

XY	AO-0 m	AO-200 m	IO-0 m	IO-200 m	PO-0 m	PO-200 m	AO[a]	IO[a]	PO[a]	Surface[b]	UTZ[b]
AO-0 m	–	0.2014	*0.0127*	0.5947	*0.0007*	0.6829	–	–	–	–	–
AO-200 m	0.0107	–	0.1150	0.3659	0.8784	*0.0181*	–	–	–	–	–
IO-0 m	*0.0256*	0.1011	–	0.5738	0.0162	0.2414	–	–	–	–	–
IO-200 m	0.1837	0.0319	0.0034	–	0.3605	*0.0152*	–	–	–	–	–
PO-0 m	*0.0000*	0.0001	0.1326	0.0441	–	*0.0023*	–	–	–	–	–
PO-200 m	0.5077	*0.0000*	0.0825	*0.0000*	*0.0005*	–				–	–
AO	–	–	–	–	–	–	–	0.4586	0.4023	–	–
IO	–	–	–	–	–	–	0.0315	–	0.0005	–	–
PO	–	–	–	–	–	–	0.8550	0.5925	–	–	–
Surface	–	–	–	–	–	–	–	–	–	–	0.5457
UTZ	–	–	–	–	–	–	–	–	–	0.1998	–

Values are showed in italics when both P_{XY} and P_{YX} are less than 0.05

[a] All surface and upper twilight zone clones were pooled for this analysis

[b] All surface or upper twilight zone (UTZ) clones were pooled for this analysis

At the subgroup level, no subgroup was detected simultaneously in all six clone libraries. *Erythrobacter*-like group I was present in all surface libraries and no subgroup was shared among three upper twilight zone libraries. For the surface and upper twilight zone libraries at the same station, common subgroups existed, e.g., *Unknown* group I in the Pacific Ocean station; *Gamma-proteobacteria*-like group III in the Atlantic Ocean station; *Unknown* group III, V, and VI, *Gamma-proteobacteria*-like group IV, *Sphingomona*-like group I, and *Roseobacter*-like group IV in the Indian Ocean station. In comparison, subgroups were most aboundant in the Indian Ocean station (seven for 0 m and eight for 200 m).

Representative sequences in each subgroup were subjected to a search for relatives in public databases (Table 5). The closest neighbors in GenBank shared 79–99% identity with our sequences and their source environments covered various marine habitats, including estuary, coast, coastal ocean, open ocean, marine animal, and marine sediment (Table 5). More than 50% of the representative clones were closely related to their neighbors from relatively oligotrophic coastal and open oceans. In the HOT metagenome database, only one read (HOT_READ_85742317) in the 70 m fraction was closely related to six sequences we obtained with the identity ranging from 79 to 96%. In the GOS metagenome database, the best match was from the hypersaline lagoon (station GS033, 13 matches, 81–89% identity). The metagenomes in the North American East Coast (st. GS013), Floreana Island coastal seawater (st. GS027), and Gulf of Mexico (st. GS016) were also found to have a few matches with the identity of 79–84%. No significant relative was found in the Mediterranean Bathypelagic Habitat (DEEPMED) and Sargasso Sea metagenomes.

Discussion and conclusions

The AAPB represent a functional bacterial group defined by their aerobic, heterotrophic, and photosynthetic abilities. Marine AAPB populations appear to be a very dynamic part of the marine microbial community as revealed by previous comprehensive investigations (Jiao et al. 2007; Yutin et al. 2007). Furthermore, due to their bigger size than normal heterotrophic bacteria (Schwalbach and Fuhrman 2005; Jiao et al. 2007; Lami et al. 2007) and rapid growth (Koblizek et al. 2007), they may contribute 4–50% of the total bacterial production in oligotrophic oceans and thus are a large sink for dissolved organic matter (Koblizek et al. 2007). Here we examined AAPB community structures in the Pacific, Atlantic and Indian Oceans, and retrieved abundant *pufM* gene fragments from the upper twilight zones in different oceanic regions, shedding new light on bacterial photoheterotrophic lifestyles in rarely explored twilight zones.

New insights into AAPB biogeography in global oceans

Culture-dependent and -independent approaches have been used to investigate AAPB diversity in natural environments. Cultivated marine AAPB reported to date are affiliated with 12 genera of *Alpha*-proteobacteria: *Roseobacter* (Shiba 1991; Allgaier et al. 2003; Green et al. 2004; Oz et al. 2005), *Erythrobacter* (Shiba and Simidu 1982; Koblizek et al. 2003), *Citromicrobium* (Yurkov et al. 1999), *Dinoroseobacter* (Biebl et al. 2005b), *Roseovarius* (Biebl et al. 2005a), *Hoeflea* (Biebl et al. 2006), *Stappia*, *Pannonibacter*, *Roseibium*, and *Labrenzia* (Suzuki et al. 2000; Biebl et al. 2007), and *Ruegeria* and *Roseovarius* (Green et al. 2004), together with a recently cultivated

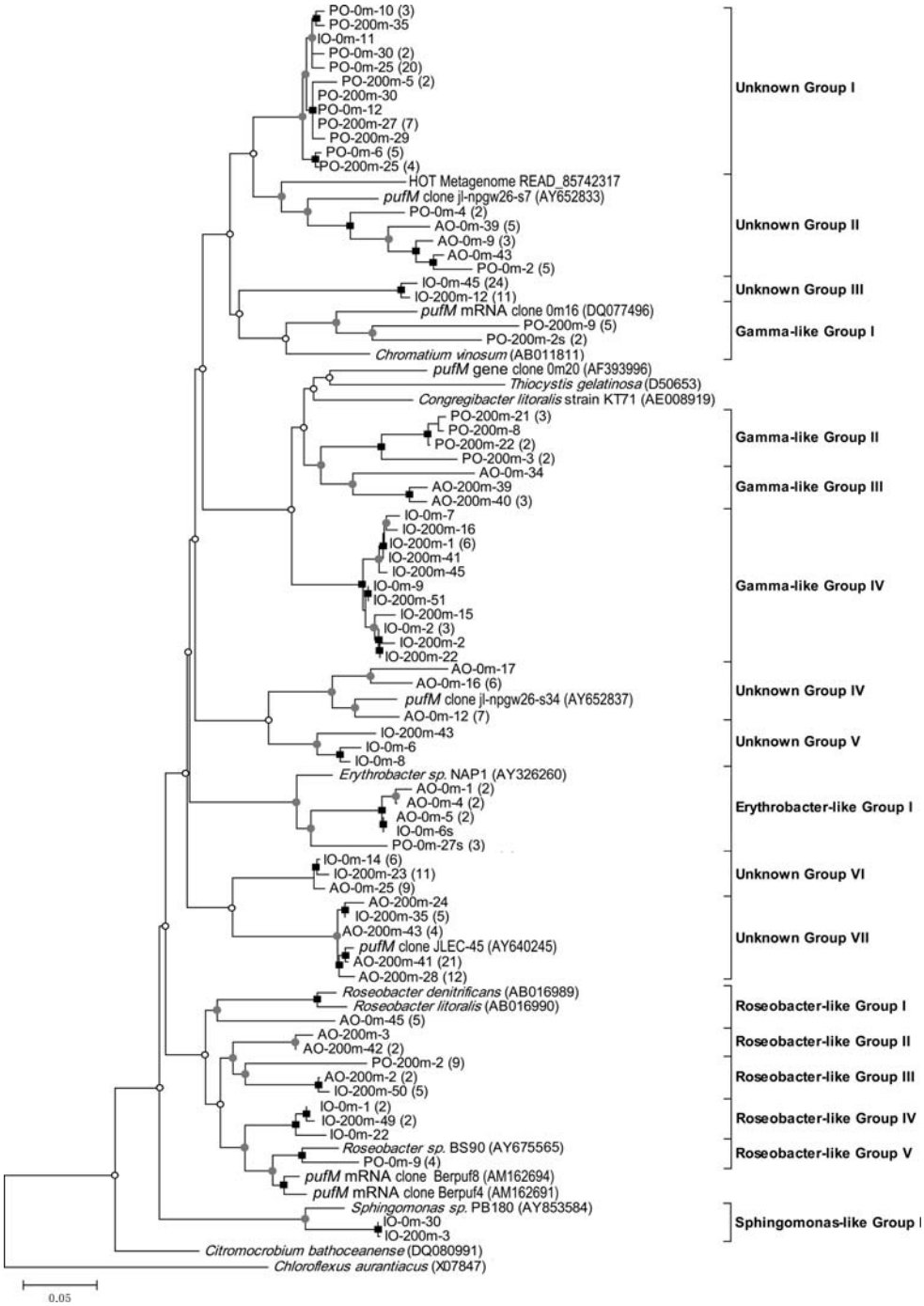

Fig. 2 Phylogenetic analysis of partial *pufM* sequences from the Pacific, Atlantic, and Indian Oceans. The phylogenetic tree was constructed with a neighbor-joining algorithm. Symbols "*filled square*", "*filled circle*", and "*open circle*" represent nodes with bootstrap values (1,000 replicates) ≥75%, ≥50%, and <50%, respectively. All distinct *pufM* sequences obtained in this study that show ≥1% nucleotide difference and reference sequences from cultures and the most similar database records were included. Scale bar represents 5% nucleotide substitution percentage. *PufM* sequence from *Chloroflexus aurantiacus* was used as the outgroup. For each library, the number of identical or almost identical (<1% nucleotide difference) sequences is listed in parenthesis

Gamma-proteobacteria AAPB, *Congregibacter litoralis* strain KT71 (Fuchs et al. 2007). Among these genera, only *Erythrobacter* and *Roseobacter*-like isolates have been reported frequently in coastal and offshore AAPB communities; whereas in oligotrophic open oceans, reports of AAPB isolation are very few. Koblizek et al. (2003) isolated six *Erythrobacter* sp. strains from the open ocean in the NE Pacific and SE and NW Atlantic. However, these stations were far away from equatorial central open oceans and thus could not represent typical oligotrophic marine regimes. Ongoing AAPB isolation works in the same stations as our study show that no AAPB have been isolated from normal marine bacterial media 2216E and RO cultures (Jiao et al., unpublished data). This suggests that various ecotypes, such as eu-/meso-/oligo-trophic AAPB species may exist in natural AAPB populations.

Fortunately, culture-independent molecular methods have extended our knowledge of AAPB diversity. In contrast to culturing data, we revealed various AAPB subgroups in the central and extremely oligotrophic open

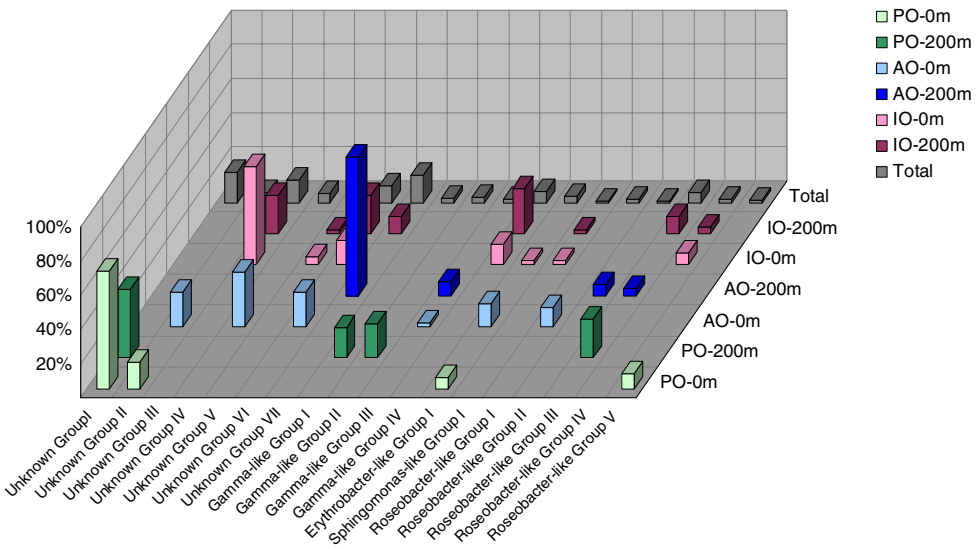

Fig. 3 Percentage abundance of *pufM* sequences in each library and phylogenetic subgroup

oceans, covering *Alpha-* and *Gamma*-like proteobacteria. Our results support the suggestion that AAPB diversity may extend beyond the two readily cultured genera *Erythrobacter* and *Roseobacter* proposed by Cottrell et al. (2006) based on the difference between FISH and microscopic IR data and by Beja et al. (2002) and Waidner and Kirchman (2005) based on environmental *puf* operon large-insert clone analysis. Our data also confirm that AAPB populations vary significantly between different oceanic regions and represent a dynamic component of the marine bacterioplankton (Jiao et al. 2007; Yutin et al. 2007).

Though much is known about natural AAPB population composition, the issue of AAPB subgroup biogeography remains controversial. *Gamma-proteobacteria* like AAPB represented a widely distributed subgroup in the China seas, the Bering sea, the Pacific (Hu et al. 2006; Jiao et al. 2007), and the Atlantic but not the Indian Ocean (Jiao et al. 2007). In this study, we retrieved abundant *Gamma-proteobacteria*-like clones (14.2% of total clones) from the surface and below euphotic zones of the Pacific and Atlantic Oceans as well. However, in another report by Yutin et al. (2007), only one station near the Galapagos Island among a total of 18 open ocean GOS stations and eight coastal stations in the North-western Atlantic or off the Galapagos Islands were found to accommodate *Gamma-proteobacteria*-like AAPB in a minor fraction. In the surface Baltic Sea, the AAPB community is reported to be dominated by *Gamma-proteobacteria* (70–75% in total clones) in July and October (Masin et al. 2006). Similarly, *Gamma-proteobacteria*-like *pufM* genes are thought to comprise at least 50% of total clones in the Delaware River (Waidner and Kirchman 2008). *Erythrobacter*-like subgroups also showed an inconsistent distribution pattern among 41 GOS stations (no fragments belonging to this subgroup obtained; Yutin et al. 2007) and those stations in the East China Sea, the South China Sea, and the Pacific Ocean (up to 25% of total clones; Jiao et al. 2007), the Baltic Sea (18% of total clones; Masin et al. 2006), the Delaware River (up to 27%; Waidner and Kirchman 2008), and the three oceans investigated in this study (3.7% of total clones).

This significant difference in the AAPB subgroup distribution pattern in global oceans may be partially explained by the presence of mesotrophic and oligotrophic *Gamma-proteobacteria*-like AAPB ecotypes (Hu et al. 2006) or habitat-specific types (Waidner and Kirchman 2008). However, inconsistent with the ecotype hypothesis, some highly similar or near identical *pufM* fragments were retrieved from different layers or stations in this study, e.g., clones PO-0 m-12, PO-200 m-27, and IO-0 m-11 in *Unknown* Group I, and clones AO-0 m-5 and IO-0 m-6 s in *Erythrobacter*-like Group I. More data are needed to clarify whether they are from the same species or just the result of horizontal gene transfer among different AAPB populations.

In addition to the systematic comparison of AAPB diversity in the three oceans, more important of this study is that we were the first to retrieve abundant *pufM* fragments from ocean depths below the euphotic zone. To date, depth data based on BChl.*a* detection showed that AAPB were most abundant in photic zones (Cottrell et al. 2006; Jiao et al. 2007; Lami et al. 2007; Zhang and Jiao 2007). In deep waters, weak signals have been detected at some stations in the Sargasso Sea (294 m; Sieracki et al. 2006), the central North Pacific, the Atlantic and Indian oceans (200 m; Jiao et al. 2007), and the central South Pacific (270 m; Lami et al. 2007), indicating the universal presence of AAPB in upper twilight zones. In contrast, no corresponding diversity data in these depths have been reported and thus direct genetic evidences is lacking to support their presence. By sampling a size range of bacterioplankton (0.22–200 μm) covering big AAPB cells and those attached to particles

Table 5 Similarity of representative *pufM* sequence in each subgroup with public database records

Subgroup	Clone	GenBank				HOT Metagenome				GOS Metagenome				
		Closest neighbor				Match sequence				Match sequence				
		id	Accession no.	Sequence identity (%)	Habitat type	Length (bp)	id	Identity (%)	Layer (m)	Length (bp)	id	Identity (%)	Station	Source region
Unk. I	PO-0 m-12	*pufM* clone 1G02 Delaware estuary	EU191257	86	Estuary	139	HOT_READ_85742317	84	70	192	EK714403	86	GS033	Hypersaline Lagoon
Unk. II	AO-0 m-43	*pufM* clone jl-npgw26-s7 North Pacific Gyre	AY652833	96	Open ocean	161	HOT_READ_85742317	96	70	192	NA	82	GS013	North American East Coast
Unk. III	IO-200 m-12	*pufM* clone JL-ECSP6-8m51 East China Sea	AY652818	84	Coastal ocean					192	EK714403	83	GS033	Hypersaline Lagoon
Unk. IV	AO-0 m-16	*pufM* clone jl-npgw26-s34 North Pacific Gyre	AY652837	95	Open ocean					192	EK714403	84	GS033	Hypersaline Lagoon
Unk. V	IO-0 m-6	*pufM* clone JL-ECSP6-8m112 East China Sea	AY652831	86	Coastal ocean	100	HOT_READ_85742317	86	70	192	EK714403	88	GS033	Hypersaline Lagoon
Unk. VI	IO-200 m-23	*pufM* clone JL-ECSP6-8m66 East China Sea	AY652823	90	Coastal ocean					192	EK714403	84	GS033	Hypersaline Lagoon
Unk. VII	AO-200 m-41	*pufM* clone JLEC-45 East China Sea	AY640245	99	Coastal ocean					192	EK714403	86	GS033	Hypersaline Lagoon
Gam.-like I	PO-200 m-8	*pufM* mRNA clone 0m16 Monterey Bay	DQ077496	95	Bay	113	HOT_READ_85742317	82	70	187	EJ109915	84	GS027	Floreana Island coastal seawater
Gam.-like II	PO-200 m-9	isolate JL351 *pufM* gene South China Sea	DQ484038	79	Open ocean					192	EK714403	83	GS033	Hypersaline Lagoon
Gam.-like III	AO-200 m-39	*pufM* clone npg-jlw26-27 North Pacific Gyre	DQ093233	83	Open ocean					192	NA	79	GS016	Gulf of Mexico
Gam.-like IV	IO-200 m-41	*pufM* gene clone 0m20 Moss Landing, California	AF393996	95	Coastal ocean	161	HOT_READ_85742317	79	70	192	EK714403	83	GS033	Hypersaline Lagoon
Ery.-like I	AO-0 m-4	*pufL/M* clone JAP23BTW Marine tidal water, India	AM944085	83	Coastal seawater					192	EK714403	81	GS033	Hypersaline Lagoon
Sph.-like I	IO-200 m-3	*pufM* mRNA clone 1F10 Delaware estuary	EU191568	87	Estuary					192	EK714403	86	GS033	Hypersaline Lagoon
Ros.-like I	AO-0 m-45	*pufM* clone cys 19, associated with ascidian	DQ858469	85	Marine animal					192	EK714403	85	GS033	Hypersaline Lagoon
Ros.-like II	AO-200 m-42	*pufM* clone jl-npgw26-s57 North Pacific Gyre	AY652839	86	Open ocean					192	EK714403	89	GS033	Hypersaline Lagoon
Ros.-like III	IO-200 m-50	*pufM* mRNA clone Berpuf13, Lagoon Mediterranean sea	AM162699	89	Marine sediment					192	EK714403	85	GS033	Hypersaline Lagoon
Ros.-like IV	IO-0 m-1	*pufM* mRNA clone Berpuf8, Lagoon Mediterranean sea	AM162694	94	Marine sediment	100	HOT_READ_85742317	88	70	192	NA	83	GS013	North American East Coast
Ros.-like V	PO-0 m-9	*pufM* mRNA clone Berpuf4, Lagoon Mediterranean sea	AM162691	95	Marine sediment					192	EK714403	81	GS033	Hypersaline Lagoon

Hawaii Ocean Time-series (HOT) depths and Global Ocean Sampling (GOS) expedition metagenomes data were blasted within the marine microbial community metagenome datasets (CAMERA) database. The algorithm BlastX in GenBank and CAMERA was used

Unk. Unknown Group, *Gam.-like Gamma-proteobacteria*-like Group, *Ery.-like Erythrobacteri*-like Group, *Sph.-like Sphingomonas*-like Group, *Ros.-like Roseobacter*-like Group

(Du et al. 2006; Waidner and Kirchman 2007), we successfully amplified out *pufM* fragments by means of nested PCR from upper twilight zones samples. However, in accordance with above AAPB abundance surveys, we failed to amplify *pufM* from greater depths (500 m and below), suggesting that AAPB distribution may be limited to upper twilight zones. In comparison to surface stations, AAPB populations in upper twilight zones had low diversity but high dominance, covering major subgroups found in surface waters. In a previous report by Jiao et al. (2007), AAPB genetic diversity is found to decrease along an environmental gradient with increasing Chl.*a* concentration. However, a similar trend was not observed in the depth data in this study since Chl.*a* concentration was below the detection limit in the upper twilight zones and AAPB diversity was relatively low. More factors may affect the AAPB diversity pattern. An interesting observation is that surface and deep stations in the Indian Ocean shared the maximum number of subgroups (6 out of a total 18, Fig. 3) among three oceans. This pattern may be related to the deeper mixed layer in the Indian Ocean than other oceans caused by monsoon winds and solar heating there (Kara et al. 2003). However, our current data are insufficient for reaching conclusions on this point and more temporal and spatial diversity data are necessary.

Adaptability and evolutionary implications of AAPB in upper twilight zones

AAPB are thought to originate from an anaerobic anoxygenic ancestor, the purple photosynthetic bacteria (Beatty 2002), which first appeared approximately 3.5 Ga (Des Marais 2000). During their long evolutionary history, AAPB may have adapted to various aerobic aquatic environments and become distributed from seawater to freshwater ecosystems and from ultra-oligotrophic oceans to eutrophic coasts. Our diversity data combined with previous BChl.*a* data in deep ocean waters is evidence for the existence of deep AAPB types, but how they adapted to, and evolved in, deep oceans is an enigma.

From the aspect of physical and chemical conditions in upper twilight zones, we attempted to correlate specific AAPB physiological properties (heterotrophic, aerobic, and photosynthetic) with environmental features and discussed the basis for AAPB existence there. Heterotrophic bacteria have been observed over the whole water column in the open ocean (Karner et al. 2001). Marine snow and animal carcasses could supply abundant organic substrate for bacteria to fulfill their heterotrophic ability. One challenge for AAPB is that mineralization of dissolved organic matter and lack of oxygenic phototrophs create a micro-aerobic environment there. However, two micro-aerobic AAPB strains have been isolated from surface seawater (Fuchs et al. 2007) and from a marine sediment surface layer (Shiba 1991), which confirm that AAPB have the ability to survive the low oxygen in upper twilight zones.

Photosynthesis is the key property that determines the success of AAPB in upper twilight zones. It is frequently argued whether extremely dim light could support photosynthesis. In the visible light spectrum, only blue can penetrate into deep waters and reach the bottom of euphotic zones and upper twilight zones with its 99% absorption occurring at 254 m in open oceans (Trujillo and Thurman 2004). The oxygenic phototroph *Prochlorococcus* has adapted to absorbing the blue part of the spectrum by means of its unique photosynthetic apparatus (Partensky et al. 1999) and is found at deep layers down to 300 m (Overmann and Garcia-Pichel 2002). In the green sulfur bacteria, Beatty et al. (2005) discovered a previously unknown species, GSB1, from deep-sea hydrothermal vents. An AAPB species, *Citromicrobium bathyomarinum*, has also been isolated from deep-sea black smoker plumes (about 2,000 m; Yurkov et al. 1999), where the only light source was geothermal radiation. These findings indicate that microbial phototrophs in diverse taxa have successfully colonized dimly-lit marine domains. These phototrophic microorganisms contained (bacterio-)chlorophylls *a*, *b*, *c*, *d*, or *e*, all of which have two absorption bands, one in the red and another in the blue (blue-green), and can broaden the spectrum of light available for photosynthesis by adjusting the positions of (bacterio-)chlorophylls in light-harvesting complexes or the components of accessory photosynthetic pigments (Jeffrey et al. 1997). These self-adjusting abilities strongly support the possibility that a blue light ecotype of AAPB is present in upper twilight zones.

During long-term adaptive evolution, surface and deep AAPB species may be imprinted by some clues in their genomes. Here we observed slightly lower GC content in deep AAPB *pufM* fragments than those from the surface. The GC contents (57.1–63.1%) we measured were close approximations to those determined from sequenced genomes of three typical AAPB species (*Roseobacter litoralis* Och 149, 57.3%; *Erythrobacter litoralis* HTCC 2594, 63.1%; *Congregibacter litoralis* KT71, 57.7%; data retrieved from NCBI genome online database). The genome GC content is suggested to be positively correlated with optimal growth temperature in various prokaryotes (Musto et al. 2006). Whether such a GC difference in photosynthetic gene fragments is also a kind of genomic adaptation of the deep AAPB to selective pressures by cold water needs to be further studied. Undoubtedly, ongoing genome sequencing of more AAPB strains will shed new light on their adaptation mechanisms and evolution in the deep oceans.

In conclusion, AAPB genetic diversity was revealed for the first time in upper twilight zones by introducing nested

PCR into *pufM* gene fragment amplification. Various subgroups were found in AAPB populations in extremely oligotrophic open oceans, covering *Alpha-* and *Gamma-proteobacteria*-like subgroups. Compared with surface stations, deep AAPB communities showed slightly lower diversity and higher dominance and a lower GC content in amplified *pufM* gene fragments. These distinct differences between surface and upper twilight zone AAPB populations imply that AAPB have adapted to both warm and sufficiently illuminated surface waters and cold and dim deep waters through co-evolution with their surrounding environment.

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