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# Real-time PCR for quantification of aerobic anoxygenic phototrophic bacteria based on *pufM* gene in marine environment

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**Abstract**

The abundance of aerobic anoxygenic phototrophic bacteria (AAPB), a new functional group that plays important roles in marine carbon cycling, is determined frequently by infrared epifluorescence microscopic analysis (IREM) or high-performance liquid chromatography (HPLC) based on detecting BChl *a* (bacteriochlorophyll *a*) fluorescence signal at 880 nm. Unfortunately, the fluorescence signal is often influenced by environmental variables and physiological state of cell. Here we developed a real-time quantitative PCR (qPCR) assay based on *pufM* gene to specifically quantify AAPB in marine environments. High specificity and sensitivity for estimation of AAPB abundance were revealed by analysis of amplification products, melting curves and target sequences. The phylogenetic tree indicated that this primer set is suitable for a wide genetic diversity of AAPB, including α-3, α-4 Proteobacteria and clones of unclear taxonomic position. In contrast, no amplicon was obtained from green non-sulphur bacteria and oxygenic phototrophic bacteria such as Cyanobacterial genomic DNA. The melting behavior could indicate predominant phenotypes in AAPB community in addition to validating the products of qPCR. The AAPB was estimated to range from $1.3 \times 10^4$ cell/ml to $3.4 \times 10^5$ cell/ml in our 10 tested water samples by this qPCR assay. Further investigations on the abundance distribution of AAPB in marine environments using the qPCR assay may provide new insight into their ecological functions.


*Keywords:* Aerobic anoxygenic phototrophic bacteria; Genetic diversity; *pufM*; Real-time quantitative PCR

## 1. Introduction

The aerobic anoxygenic phototrophic bacteria (AAPB) are a new functional group capable of producing photoinduced electron transport only under aerobic conditions (Rathgeber et al., 2004). AAPB are widely distributed in the euphotic zone and account for $2 \pm 5\%$ of the photosynthetic electron transport and at least 11% of the total microbial community in the upper ocean. AAPB has been suggested to be critical to the cycling of both organic and inorganic carbon in the ocean due to their obligate aerobic anoxygenic photosynthetic functions (Kolber et al., 2000, 2001). Furthermore, AAPB are capable of controlling the expression of their photosynthetic apparatus BChl *a*; i.e., they are facultative phototrophs switching to heterotrophic metabolism with less BChl *a* (bacteriochlorophyll *a*) synthesis under organic-rich conditions (Kolber et al., 2001). Besides organic carbon, light and oxygen are also important environmental properties that control the accumulation of BChl *a*, while it was severely reduced in the anoxic condition or the presence of dim light (Yurkov and van Gemerden, 1993a,b; Suyama et al.,

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2002). Several BChl-free strains of *Roseovarius tolerans* begin producing BChl *a* only after 6 years of subculturing (Labrenz et al., 1999), which indicates that unknown factors may influence the activation of photosynthesis genes in AAPB, and suggests that some members of AAPB may be overlooked due to low or variable production of BChl *a*.

Thus the absolute or relative abundance within the microbial community based on BChl *a* fluorescence signal at 880 nm measured by infrared epifluorescence microscopic (IREM) and high-performance liquid chromatography (HPLC) are inevitably influenced by the environmental properties (Kolber et al., 2000, 2001). In addition, the recent work of our group found that IREM technology could introduce false positive results in AAPB enumeration due to infrared fluorescence emission from other microbes especially Cyanobacteria, whose abundance is comparable to that of AAPB. And then we developed a Cyanobacteria-calibrated Infrared Epifluorescence Microscopy (ccIREM) method and revealed that such errors were about 30% in coastal waters and could even be higher in oligotrophic oceanic waters where Cyanobacteria are more abundant (Zhang and Jiao, 2003). Thus approaches independent of intracellular BChl *a* contents are important for direct estimation of the abundance of AAPB in marine environments.

Recent molecular phylogenetic analyses based on *pufM* gene and 16S rRNA gene sequencing have revealed a wide phylogenetic diversity of AAPB containing α-Proteobacteria, β-Proteobacteria related clusters and even distantly related photosynthetically active bacterial groups (Beja et al., 2002; Koblizek et al., 2003). The 16S rRNA gene was not an appropriate target for specific primers to be used for quantification AAPB due to their phylogenetic diversity and high variation in copy number among species. Real-time quantitative PCR offers high sensitivity and specificity to trace and quantify the PCR products formed during the exponential phase of the reaction. The detection of PCR products is monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA (dsDNA) (Higuchi et al., 1992), which can provide both quantitative results and differentiation of PCR products by analysis of melting curves (Ririe et al., 1997).

In this paper, we developed a real-time quantitative PCR (qPCR) assay based on partial sequence of *pufM* gene encoding M subunit of the photosynthetic reaction center complex of anoxygenic phototrophic bacteria. This assay is capable of quantifying AAPB independent of intracellular pigment concentrations.

## 2. Materials and methods

### 2.1. Sampling sites

We sampled the surface water (0–1 m) from the Yangtze River estuary (122.500–123.500W; 29.001–32.000N) on August in 2002, the East China Sea (122.84–129.00E; 31.00–32.00N) during September–October in 2003, South China Sea on February in 2004, and Xiamen Bay in Taiwan straits on May in 2004. Ten out of all the samples were selected according to their distances offshore in this qPCR assay.

### 2.2. Samples collection and community DNA extraction

Water samples (3–10 l) for DNA extraction were pre-filtered through a 200-μm mesh and subsequently filtered onto a 0.22 μm pore size filter (Pall, Gelman Sciences Inc). Filter samples were immediately frozen in liquid nitrogen and then transferred to −80 °C for storage. In order to confirm whether AAPB-attached particles can be removed by pre-filtering seawater through <3 μm filters as employed in previous reports (Beja et al., 2002), 6 l of seawater in triplicate were collected from the surface coastal water at station XM of the Taiwan straits and filtered through a 3 μm pore size filter, 0.22 μm pore size filter after pre-filtered through a 200-μm mesh, and 0.22 μm pore size filter after pre-filtered through 3 μm pore size filters (Pall, Gelman Sciences Inc.), respectively. All three kinds of filter samples were preserved for quantification of AAPB using qPCR. Community DNA was extracted using the hot SDS, phenol/chloroform/isoamyl alcohol, ethanol precipitation extraction protocol initially described by Fuhrman et al. (1988) and then purified twice. DNA quality and size was checked by 1% (wt/v) agarose gel electrophoresis and spectrophotometer at 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). Purified DNA and DNA in community with crude extract (obtained before phenol/chloroform/isoamyl alcohol treatment) were quantified using PicoGreen dsDNA quantification kit (Molecular Probes) and spectrofluorophotometer RF-5301PC (Shimadzu). The recovery rates of DNA extraction were determined by dividing the mass of final purified DNA by the mass of DNA in community with initial crude extract.

### 2.3. Primers for pufM gene

The partial fragment (193 bp) of *pufM* gene was amplified with the primers set originally described in Beja et al. (2002) and Achenbach et al. (2001), which is