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Novel Primers Reveal Wider Diversity among Marine Aerobic Anoxygenic Phototrophs†

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Aerobic anoxygenic phototrophic bacteria (AAnPs) were previously proposed to account for up to 11% of marine bacterioplankton and to potentially have great ecological importance in the world's oceans. Our data show that previously used primers based on the M subunit of anoxygenic photosynthetic reaction center genes (*pufM*) do not comprehensively identify the diversity of AAnPs in the ocean. We have designed and tested a new set of *pufM*-specific primers and revealed several new AAnP variants in environmental DNA samples and genomic libraries.

Recent reports suggested that bacteriochlorophyll *a* (BChl*a*)-containing aerobic anoxygenic phototrophic bacteria (AAnPs) comprise a significant fraction of marine bacterioplankton communities, representing up to 11% of the total surface water microbial community, and thus potentially have a great ecological importance in the world's oceans (6, 7). *pufM* genes (encoding the M subunit of anoxygenic photosynthetic reaction centers) were recently used to assess the diversity of different aerobic anoxygenic photosynthetic assemblages (2, 3, 12). These studies show that *Roseobacter* and *Roseobacter*-like bacteria constitute a significant proportion of AAnPs in the oceans (3, 12).

The relative abundance and importance of AAnPs to the flow of energy and carbon in the ocean are still controversial. Using infrared epifluorescence microscopy and real-time PCR, Schwalbach and Fuhrman (15) suggested that AAnPs make up a small portion of the total prokaryotic cells in the upper ocean (up to 2.2%). Furthermore, a study by Goericke (4) using BChl*a* measurements suggested that the contribution of BChl*a*-driven anoxygenic bacterial photosynthesis in the ocean to light-energy conversion is substantially smaller than the previously suggested 5 to 10% global average (6, 7). Since PCR-based studies depend on the ability of primers to target diverse sequences, we decided to test the efficacy of the most widely used *pufLM* primer set, originally designed by Nagashima and coworkers (10) and used in the original form (1, 5, 15) or with slight modifications (3, 12) for the amplification and quantification of *pufLM* and *pufM* genes directly from the environment.

To date, all previously published primers targeting *pufL* and *pufM* (1, 3, 5, 10, 15, 16) were designed based on nucleotide sequences of known *pufLM* genes. Primers designed based on nucleotide alignments have an inherently smaller number of degeneracies than those designed based on amino acid alignments. These primers possibly miss sequences representing alternative codons to particular amino acids. We aligned over 200 *pufM* nucleotide sequences available in GenBank and compared the sequences of the current *pufM* primers to their target regions in the alignment (see Table S1 in the supplemental material). One such comparison, performed for the pufM_rev primer published by Nagashima et al. (10), is shown in Table 1. We chose this primer for a more detailed analysis since it was used (with slight modifications) in almost all previous studies retrieving these genes by PCR. For the same reason, most *pufM* sequences available in GenBank did not contain this priming region, and thus only 33 sequences from GenBank are presented in Table 1. The pufM_rev primer matches most of the sequences that originated from cultured strains, which is not surprising since these sequences were used to design the primer. Moreover, no differences in codon usage were observed (i.e., identical protein fragments were encoded by the same nucleotide sequences). Therefore, sole targeting of these sequences would not require an amino acid-based degenerate primer.

Environmental *pufM* fragments show much greater variability in codon usage for the same amino acids (Table 1). Furthermore, nearly all differences in nucleotide sequences represent silent mutations (shown in bold) and do not affect the consensus protein sequence. The mismatches between environmental sequences and primer pufM_rev clearly show that better and more general primers are needed to uncover the diversity of marine AAnPs.

We therefore designed new primers based on an amino acid alignment of PufM proteins, including all possible degeneracies. The best-conserved regions of the protein alignment were located near the same positions as the previously used pufM_fwd and pufM_rev primers (1, 3). These regions were used to design new primers named pufM_uniF (GGNAAYYTNTWYTAYAAYCCNTTYCA) and pufM_uniR (YCCATNGTCCANCKCCARAA) (Fig. 1; Table 2). In addition, using an alignment of translated environmental genomic and shotgun sequences, a well-conserved region downstream of pufM_rev was found and used to design a second protein-based reverse primer, named pufM_WAW (AYNGCRAACCACCANGCCCA). We used primer pairs pufM_uniF plus pufM_uniR and pufM_uniF plus pufM_WAW to amplify a number of new *pufM* fragments from environmental DNA samples as well as from bacterial artificial

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† Supplemental material for this article may be found at http://aem.asm.org/.

TABLE 1. pufM_rev primer compared to its corresponding region in aligned *pufM* sequences

PufM_rev primer (complement strand) for:	Codon encoding amino acid[a]							No. of mismatches
	F	W	R	W	T	M	G	
	T T C	T G G	C G C	T G G	A C C/G	A T G	G G	
Cultured bacteria								
Acidiphilium cryptum	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
Bradyrhizobium ORS278	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
Chloroflexus aurantiacus	T T C	T G G	C G C	T G G	t g C	A T G	G G	2
Ectothiorhodospira shaposhnikovii	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
Jannaschia CCSI	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
Lamprocystis purpurea	T T g	T G G	C G C	T G G	A C C	A T G	G G	1
Rhodobacter azotoformans	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
Rhodobacter capsulatus	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
Rhodobacter sphaeroides	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
Rhodobacter veldkampii	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
Rhodospirillum molischianum	T T C	T G G	C G C	T G G	c a G	c T t	G G	4
Rhodospirillum rubrum	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
Rhodovulum sulfidophilum	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
Roseateles depolymerans	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
Roseiflexus castenholzii	T T C	T G G	C G C	T G G	g t G	A T G	G G	2
Roseospirillum parvum 9301	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
Rhodopseudomonas palustris	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
Blastochloris viridis	T T C	T G G	C G C	T G G	A C G	A T C	G G	0
Rubrivivax gelatinosus	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
Environmental sequences[b]								
DelRiver_fos13D03*	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
DelRiver_fos06H03*	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
EBAC000_29C02**	T T C	T G G	**A** G **A**	T G G	A C C	A T G	G G	2
EBAC000_60D04**	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
EBAC000_65D09**	T a C	T G G	C G C	T G G	t g C	A T G	G G	3
eBACred25D05***	T T C	T G G	C G C	T G G	A C **A**	A T G	G G	1
IBEA_CTG_UAAQ029TR	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
IBEA_CTG_SZAFS75TR	T T **T**	T G G	C G C	T G G	A C **T**	A T G	G G	2
IBEA_CTG_SSBKC12TF	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
IBEA_CTG_SSAYW76TF	T T C	T G G	**A** G **A**	T G G	A C C	A T G	G G	2
IBEA_CTG_SKBBG42TR	T T **T**	T G G	**A** G **A**	T G G	A C **A**	A T G	G G	4
IBEA_CTG_2156731	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
IBEA_CTG_2073229	T T C	T G G	**A** G **A**	T G G	A C C	A T G	G G	2
IBEA_CTG_2058454	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
Sequences from this study								
eBACmed94_waw	T T **T**	T G G	C G **T**	T G G	A C **A**	A T G	G G	3
eBACmed88_waw	T T C	T G G	**A** G **A**	T G G	A C C	A T G	G G	2
eBACmed26_waw	T T **T**	T G G	C G **T**	T G G	A C **A**	A T G	G G	3
eBACmed53C_waw	T T C	T G G	C G C	T G G	A C G	A T G	G G	0
eBACmed49G_waw	T T C	T G G	**A** G **A**	T G G	A C **T**	A T G	G G	3
eBACmed75G10_waw	T T C	T G G	**A** G **A**	T G G	t g C	A T G	G G	4
eBACmed19_waw	T T C	T G G	**A** G **A**	T G G	A C G	A T G	G G	2
eBACmed31B01	T T **T**	T G G	C G **T**	T G G	A C **A**	A T G	G G	3
envMED_0ma_waw	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
envMED_0mb_waw	T T **T**	c G G	C G **T**	T G G	A C **A**	A T G	G G	4
envMED_0mc_waw	T T C	T G G	C G C	T G G	A C C	A T G	G G	0
envMED_12m2_waw	T T C	T G G	**A** G **A**	T G G	A C C	A T G	G G	2
envMED_S06_waw	T T C	T G G	**A** G **A**	T G G	A C C	A T G	G G	2
envRED_7m4_waw	T T C	T G G	**A** G **A**	T G G	A C C	A T G	G G	2
envRED_30m_waw	T T C	T G G	C G C	T G G	A C **A**	A T G	G G	1
envRED_50m_waw	T T C	T G G	**A** G **A**	T G G	A C **T**	A T G	G G	3

[a] Mismatches leading to missense mutations are shown in lowercase; mismatches leading to silent mutations are shown in bold.

[b] *, fosmid clones retrieved by Waidner and Kirchman (18); **, clones retrieved by Béjà et al. (3); ***, clones retrieved by Oz et al. (12); IBEA_CTG, clones from Venter et al. (17).

chromosome (BAC) clones. All PCRs were performed in a total volume of 25 μl containing 1× PCR buffer (TaKaRa Bio Inc., Shiga, Japan), 2 mM $MgCl_2$, a 0.2 μM concentration of each deoxynucleoside triphosphate, a 0.2 to 0.4 μM concentration of each primer, 1 μl of template DNA (ca. 10 ng), and 2.5 U of ExTaq DNA polymerase (TaKaRa). PCR cycling conditions were as follows: initial denaturation step at 94°C (3 min) followed by 34 to 40 cycles of denaturation at 94°C (30 s), annealing at 50°C (45 s), and extension at 72°C (30 s) and a final extension at 72°C for 10 min. Primer puf_WAW was

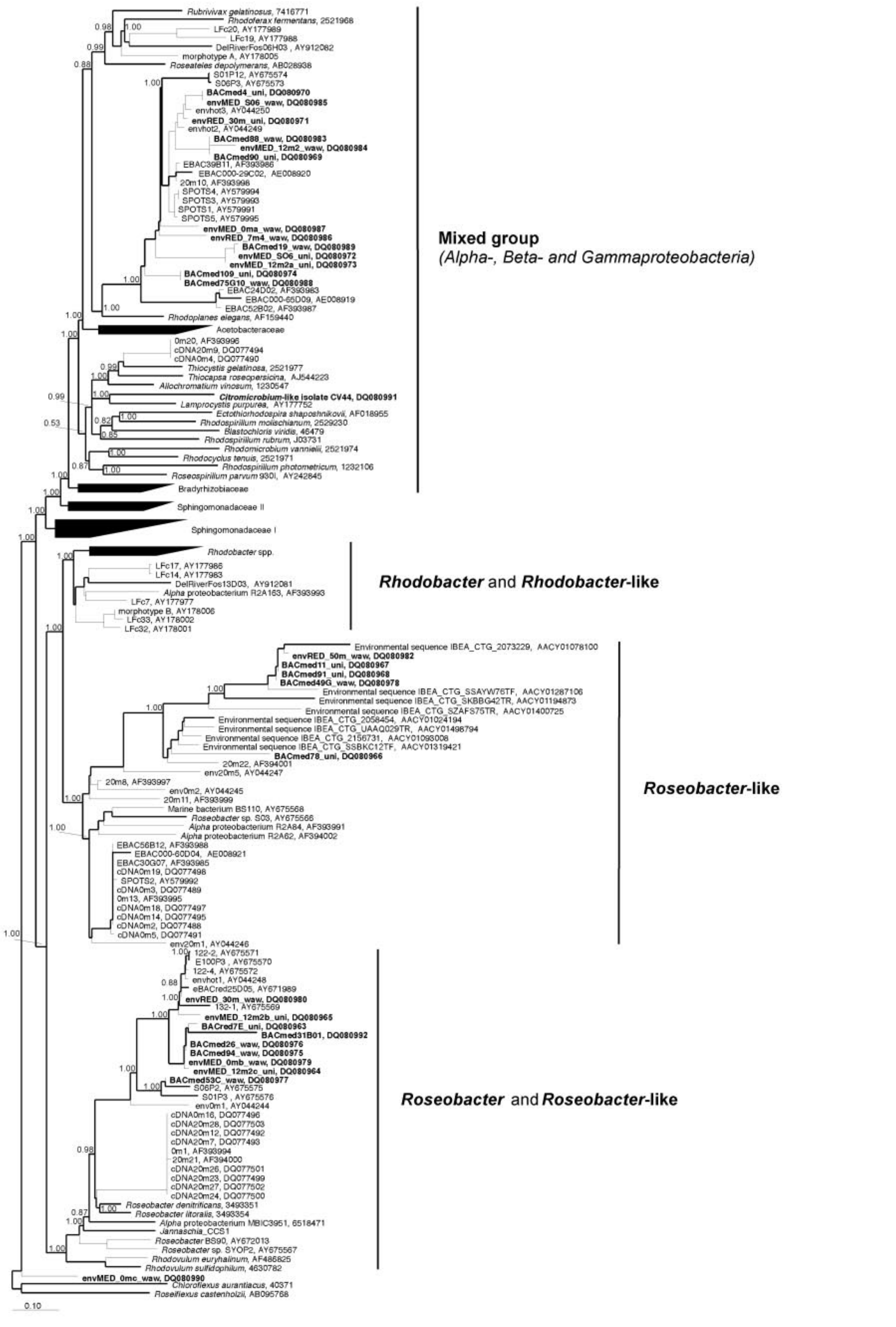

FIG. 1. *pufM* phylogenetic tree based on a Bayesian tree to which short sequences were added by ARB parsimony. The branches that appeared on the original Bayesian tree are shown with thicker lines. The numbers on nodes represent confidence values. Sequences obtained in this study are shown in bold.

TABLE 2. Efficacy of some *pufM* forward primers, measured as the number of mismatches to various *pufM* sequences only sequences containing priming regions were taken into analysis

Primer	Reference(s)	Primer length (nt)	Total no. of sequences analyzed	No. of sequences with indicated no. of mismatches				
				0	1	2	3	More than 3 or mismatched 3′ end
pufM.557F	1	16	102	6	26	36	24	10
pufM_fwd	1, 3	18	114	24	36	16	5	33
Forward primer	15	17	114	6	2	19	35	52
pufM_uniF	This study	26	114	101	11	0	0	2

used as the reverse primer with pufM_uniF to allow us to supplement the previous analysis of the pufM_rev region with new data (Table 1). As previously observed for environmental *pufM* records, these sequences have almost exclusively silent mutations in the pufM_rev priming region, and these new data clearly show that none of the codons used for the design of pufM_rev have any prevalence in the environment.

In a previous study, we were only able to detect a single *pufM*-containing clone in a BAC library prepared from the Red Sea, and no such clones were detected in a BAC library prepared from Eastern Mediterranean Sea waters (12). We used the newly designed primers (pufM_uniF and pufM_uniR) to rescreen the same Eastern Mediterranean Sea and Red Sea BAC libraries. Fourteen new *pufM*-containing BAC clones from the Eastern Mediterranean Sea library and one from the Red Sea library were found using the new primers. Additionally, 14 novel *pufM* fragments were amplified and cloned from marine DNA samples from the Mediterranean and Red seas. The collection of marine DNA samples and construction of environmental BAC libraries were described by Oz et al. (12) and Sabehi et al. (14). Seawater was prefiltered through a GF/A filter, collected on a 0.2-μm Sterivex filter, and extracted as previously described (9), and one *pufM* sequence was obtained from a *Citromicrobium*-like isolate, CV44. These sequences were combined with all *pufM* sequences previously deposited in GenBank for phylogenetic analysis, translated, and aligned using ClustalW in ARB (8) and T_Coffee (11). The resulting protein alignment was then used to realign (back translate) nucleotide sequences in ARB (8), and this nucleotide alignment was used to generate a Bayesian phylogenetic tree (Fig. 1), using a filter that excluded positions where gaps outnumbered characters and that kept the nucleotides in frame (702 positions). The Bayesian tree was generated by MrBayes 3.0 (13), using the general time reversible model and rates varying according to codon positions. Four parallel chains of 1 million generations were run, trees were sampled every 100 generations, and 1,600 "burn-in" trees were excluded from the consensus tree. This consensus tree was imported into ARB, and short sequences were added to this tree using the add-by-parsimony algorithm with the same filter.

Overall, the *pufM* diversity detected in the Mediterranean and Red seas somewhat resembles that reported for the Pacific Ocean (3), with both *Alpha*- and *Gammaproteobacteria* dominating the AAnP population. In addition, we recovered sequences grouping with *pufM* records previously retrieved only from Sargasso Sea shotgun libraries (IBEA_CTG sequences in Fig. 1) (17). No representatives from this group were found in our previous PCR-based studies (3, 12).

In previously published studies, the original *pufM* primers (1, 3) and their variants were used to uncover AAnP and anaerobic anoxygenic photosynthetic bacterial diversity in a variety of environments (2, 3, 5, 12, 16). Recently, the same primers were also used to quantify AAnP numbers via real-time PCR (15). We measured the quality of the different primers based on the total number of mismatches as well as 3′-end mismatches to a given sequence (see Table S1 and color-coded Fig. S1 in the supplemental material). Figure S1 in the supplemental material shows *pufM* phylogenetic trees based on the same tree shown in Fig. 1, indicating the suitability of the different primers for each of the sequences. Table 2 presents a short summary of the analysis shown in Fig. S1 in the supplemental material. As shown in Table 2 and Fig. S1 in the supplemental material, the primer pufM_uniF designed for this study has better coincidence and considerably fewer mismatches with environmental *pufM* sequences currently deposited in GenBank than previously utilized primers. This analysis also shows that primer mismatches might help to explain the somewhat low abundances of AAnPs estimated by real-time PCR (15). In conclusion, we believe that the newly designed primers represent a significant improvement over previously used primers and will recover a wider diversity of marine AAnPs as well as novel anaerobic anoxygenic phototrophic populations from different environments.

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