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Diversity and Distribution of Ecotypes of the Aerobic Anoxygenic Phototrophy Gene *pufM* in the Delaware Estuary▽†

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The diversity of aerobic anoxygenic phototrophic (AAP) bacteria has been examined in marine habitats, but the types of AAP bacteria in estuarine waters and distribution of ecotypes in any environment are not well known. The goal of this study was to determine the diversity of AAP bacteria in the Delaware estuary and to examine the distribution of select ecotypes using quantitative PCR (qPCR) assays for the *pufM* gene, which encodes a protein in the light reaction center of AAP bacteria. In PCR libraries from the Delaware River, *pufM* genes similar to those from *Beta-* (*Rhodoferax*-like) or *Gammaproteobacteria* comprised at least 50% of the clones, but the expressed *pufM* genes from the river were not dominated by these two groups in August 2002 (less than 31% of clones). In four transects, qPCR data indicated that the gammaproteobacterial type of *pufM* was abundant only near the mouth of the bay whereas *Rhodoferax*-like AAP bacteria were restricted to waters with a salinity of <5. In contrast, a *Rhodobacter*-like *pufM* gene was ubiquitous, but its distribution along the salinity gradient varied with the season. High fractions (12 to 24%) of all three *pufM* types were associated with particles. The data suggest that different groups of AAP bacteria are controlled by different environmental factors, which may explain current difficulties in predicting the distribution of total AAP bacteria in aquatic environments.

Several groups of bacteria potentially have the capacity to derive extra energy from light while assimilating organic matter for carbon and energy (13). Among potential photoheterotrophs are the aerobic anoxygenic photosynthetic (AAP) bacteria. The abundance of these bacteria varies greatly among aquatic regimes (0 to 20% of total bacterial abundance) (8, 21, 23, 28, 31), with estuaries having some of the highest estimates (28, 38). The reasons for this high variation are not clear, although environmental factors, such as nutrient status (21), light, and particles (38), have been hypothesized to control AAP bacterial communities. The distribution of specific groups of AAP bacteria, which has not yet been examined in depth, may provide some clues.

Culture-dependent and -independent studies suggest that there are habitat-specific types of AAP bacteria. Culture-dependent studies found isolates typical of marine and saline habitats (18), and a recent examination of metagenomic clones from the Global Ocean Sampling revealed differences in the composition of AAP bacterial communities between estuarine and oceanic regimes (41), although only a single location in the estuaries was examined. In oceanic and coastal waters, AAP bacteria belong to the alpha-3 and alpha-4 subclasses of *Alphaproteobacteria* (*Roseobacter* and *Erythrobacter*) (2, 20, 25), and PCR and metagenomic clones from coastal waters (4, 16) contain *pufM* DNA sequences closely related to those of a gammaproteobacterium in the OM60 clade (6) and to those of *Congregibacter litoralis* sp. strain KT71 (15). Phylogenetic analyses of *pufM* and other photosynthesis genes suggested that uncultured AAP bacteria related to *Rhodobacter* are abundant in the Delaware estuary (37). Additionally, 16S rRNA analyses indicate that *Rhodobacter*-like bacteria are capable of inhabiting estuarine waters with a wide range of salinities (10). One type of riverine AAP bacterium is related to strictly freshwater *Betaproteobacteria*, such as *Roseateles depolymerans* (34) and members of the *Rhodoferax* clade (11). Although the diversity of AAP bacteria is starting to become clear, we know little about estuarine ecotypes or about the distribution of ecotypes in any environment.

The aim of this work was to examine freshwater and estuarine ecotypes of *pufM* genes from uncultivated AAP bacteria in the Delaware Estuary. In the Delaware, as in other estuaries, the *Betaproteobacteria* are abundant and active in freshwater, the *Alphaproteobacteria* dominate saline habitats, and the *Gammaproteobacteria* are evenly distributed throughout the salinity gradient (5, 7, 11). We hypothesized that the distribution of specific types of AAP bacteria in the estuary would be similar to that of phylogenetic groups defined by rRNA genes. To determine the dominant types of AAP bacteria, clone libraries of *pufM* genes and transcripts from the Delaware River and Bay were constructed. Three ecotypes of *pufM* were targeted using specific quantitative PCR (qPCR) primer pairs, and the abundances of these ecotypes were examined throughout the salinity gradient of the estuary. We found that two of these ecotypes occupied specific ecological regimes repeatedly over several years, regardless of season.

MATERIALS AND METHODS

Sampling and environmental parameters. Samples were obtained on six cruises from the main stem of the Delaware estuary at an approximately 1-m depth. Nutrient concentrations were determined using a Perstorp flowthrough

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TABLE 1. Primers used in cloning and ecotype-specific qPCR of *pufM*[a]

Target	Samples	Forward primer	Reference	Reverse primer	Reference	Length (bp)[b]	Temp (°C)[c]
All *pufM* genes	River, Dec. 2001	*puf*LF (CTKTTCGACTTC TGGGTSGG)	24	*pufM*750R (CCCATGGT CCAGCGCCAGAA)	1	1,500	58
All *pufM* genes	River, Aug. 2002	*puf*LF	24	*pufM*750R	1	1,500	58
All *pufM* genes	River, Aug. 2002 cDNA	*pufM*557F (CGCACCTGG ACTGGAC)	1	*pufM*750R	1	233	58
All *pufM* genes	Bay, Aug. 2002	*pufM*557F	1	pufM_WAW (AYNGCR AACCACCANGC CCA)	40	277	56
Rhodoferax-like *pufM* genes	All qPCR samples	RfxF2 (TGGACGGCCGC ATTCTCA)	This study	RfxR2 (GCTCAATTTCG CGTTCACCACCAA)	This study	156	60
Rhodobacter-like *pufM* genes	All qPCR samples	RbaF1 (TGGACGAACCT GTTCAGC)	This study	RbaR1 (CAACTCGCGG TCGCC)	This study	152	50
gammaproteobacterial *pufM* genes	All qPCR samples	DelMGF1 (ACCGCCGCC TTCTCCAT)	This study	DelMGR1 (CTAGCTCC CGATCGCCACC ATA)	This study	151	57
16S rRNA genes, all bacteria	All qPCR samples	BACT1369F (CGGTGAAT ACGTTCYCGG)	36	PROK1541R (AAGGAG GTGATCCRGCC GCA)	36	192	60

[a] The sequence of each primer is next to the name in parentheses.
[b] PCR product length in base pairs.
[c] Annealing temperature.

analyzer using colorimetric assays as described previously (26). Temperature, oxygen, and salinity (expressed as unitless values on the practical salinity scale) were measured using a CTD 911 Plus device (SeaBird Electronics, Bellevue, WA). Detailed data on temperature, oxygen, salinity, chlorophyll, and nutrient and calculated seston concentrations are provided in Table S1 in the supplemental material.

In December 2001, DNA was isolated from free-living bacterioplankton of the Delaware River as described previously (9). In August, October, and November 2002 and July 2004, the bacterial size fraction was isolated from whole water by sequential filtration through 3.0- and 0.8-μm polycarbonate filters (147-mm diameter; Poretics). In March 2005, the bacterial size fraction was isolated as described previously (38). DNA was extracted with phenol-chloroform and further purified using the IsoQuick nucleic acid extraction kit (ISC Bioexpress, Kaysville, UT) or by cetyltrimethylammonium bromide extraction. Samples intended for RNA purification were preserved in RLT buffer with β-mercaptoethanol (Qiagen, Germantown, MD) and frozen in liquid nitrogen. Total RNA was purified on RNeasy minicolumns according to the manufacturer's recommendations (Qiagen).

Clone libraries of *pufM*. Four *pufM* libraries were constructed with nucleic acids from surface waters of the Delaware estuary (Table 1). Three samples for library construction were obtained from the river (40°7.6′N, 74°48.4′W), 198 km from the mouth of the estuary, and the bay library sample was from surface water 9 km from the mouth of the estuary (38°6.5′N, 75°6.0′W). Amplification conditions consisted of 30 cycles of denaturation (94°C for 30 s), annealing at temperatures given in Table 1 for 30 s, and extension at 72°C. Extension times for the 1,500-bp *pufLM* and 200- to 300-bp *pufM* fragments were 1 min and 30 s, respectively. All reaction mixtures contained final concentrations of reagents as follows: 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.02 U/μl *Taq* (Promega), 0.1 μM of each primer (MWG, Germany), and 50 to 200 ng template DNA. Each library was generated from a pool of three reaction mixtures which was concentrated on Microcon columns (molecular weight cutoff, 10,000) by centrifugation at 1,000 × *g* for 5 min. Pooled products were electrophoresed through SeaKem agarose (BioWhittaker, Frederick, MD), excised, and cloned into vector pCR2.1 (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions for cloning directly from low-melting-point agarose.

cDNA from the August 2002 river RNA was generated using Superscript II reverse transcriptase (RT) (Invitrogen), primed with the reverse primer *pufM*750R (Table 1). The RNA was treated with DNase I (Invitrogen) at 24°C as per the manufacturer's recommendations. The DNA-free RNA was divided into four reaction mixtures which included all reagents except the RT. Template denaturation and priming were performed as per the manufacturer's recommendations. Three reaction mixtures then received 5 U each of the RT, and the fourth reaction mixture was the no-RT control. First-strand synthesis was at 42°C for 20 min. Following PCR amplification with *pufM*557F and *pufM*750R (Table 1), the reactions were examined by gel electrophoresis to confirm that no product was generated in the no-RT control and that the correct-sized product was amplified. The three RT-PCRs were pooled and cloned as described above.

All clone libraries were screened for inserts by colony PCR with the M13 primer sequences flanking the pCR2.1 cloning site. Amplification was carried out with 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 to 90 s. After they were checked by gel electrophoresis, PCR products were purified using the QiaQuick centrifugation method and eluted in 30 μl of elution buffer (Qiagen). The clones from the August 2002 river cDNA and bay DNA libraries were sequenced using BigDye 3.1 (Applied Biosystems, CA) on a Spectrumedix 24-capillary SCE2410 apparatus. The *pufLM* products from the December 2001 and August 2002 libraries were sequenced from purified plasmid DNA using both the M13F and M13R primers.

Sequences were analyzed and edited using the SeqMan program (DNAStar, Madison, WI). Libraries of *pufLM* were checked for chimeric clones using the Bellerophon program (17). To detect chimeric clones in the *pufM* libraries, sequence alignments of the full-length and C-terminal and N-terminal regions were constructed. Phylogenetic trees were generated from the three alignments and manually compared for differences in branch patterns. Sequences of individual clones that did not branch as expected based on the full-length alignment were inspected for chimera formation. From each library, five clones or fewer were rejected as chimeras. The estimated diversities of the clones (Chao I) in the libraries were determined using the DOTUR software package (27). Comparative sequence analyses were performed using the Megalign (DNAStar), MEGA 3.1 (22), BLAST-X, version 2.2.9 (3), and batch BLAST-X (GreenGenes [greengenes.lbl.gov]) software programs.

qPCR of *pufM* ecotypes. Three primer sets were designed to amplify specific ecotypes of *pufM* in qPCR reactions (Table 1). The first type, *Rhodoferax*-like *pufM*, was designed to target genes in hypothesized representative freshwater AAP bacteria. The primers were designed to match exactly to sequence coding for the N-terminal end of the DelRiverFos06H03 product (37). The second primer pair, *Rhodobacter*-like *pufM*, was designed against an estuarine type of *pufM* in the fosmid clone DelRiverFos13D03 (37). The last set, the Delaware marine group, was designed to target a dominant group (37%) of *pufM* genes in the Delaware Bay library (August 2002) constructed in this study. To determine the abundances of all bacteria in whole water and free-living bacterial fractions, qPCR was performed using the BACT1 primer pair as described by Suzuki et al. (36). Template inhibition was checked by performing qPCR assays on serially diluted environmental DNA samples. Regardless of the DNA purification method, the efficiency of amplification ranged from 82 to 88%, as determined by the slope of the regression of logfold DNA dilution with threshold cycle values.

Amplification of all *pufM* genes was done under the following conditions: 10 min of denaturation and activation of the enzyme at 95°C, followed by 40 cycles of denaturation at 95°C (15 s), annealing at the temperatures specified in Table 1 (45 s), and extension and detection at 72°C (45 s). Reactions targeting the 16S

TABLE 2. Composition and diversity of *pufM* PCR and RT-PCR libraries from the Delaware estuary in December 2001 and August 2002[a]

Library	Total no. of clones	No. of groups	% Coverage[b]	Chao I
December 2001 river	76	33	57	65 (43, 129)
August 2002 river	95	23	76	37 (26, 79)
August 2002 river RNA	96	21	79	31 (22, 74)
August 2002 bay	107	26	76	61 (36, 152)

[a] Groups were defined at 3% amino acid sequence divergence. The Chao I index was calculated using the DOTUR software program. Numbers in parentheses are 95% confidence limits.

[b] Percent coverage was calculated with the formula $[1 - (n/N)] \times 100$, where n is the number of singleton clones and N is the total number of sequences.

rRNA gene were conducted for 30 cycles of amplification. All products were detected by an Applied Biosystems 7500 PCR system using Sybr green I fluorescence. Amplification reactions contained the following: 1× brilliant Sybr green master mix (Stratagene, La Jolla, CA), 80 pg/μl DNA, 0.096 μM (each) primer, and water to a 12.5-μl final reaction volume. All reactions were completed with a dissociation step to check for nonspecific amplification.

Standards were purified fosmid and plasmid clones. The *Rhodobacter*-like and *Rhodoferax*-like *pufM* standards were DelRiverFos13D03 and DelRiverFos06H03, respectively. The standard for the Delaware marine group (gammaproteobacterial *pufM*) was plasmid clone DB_2E03 from the general *pufM* library constructed from the August 2002 bay sample. The plasmid was linearized with NotI restriction endonuclease (New England Biolabs, Ipswitch, MA), electrophoresed through agarose, and purified with the GeneClean spin system (Bio101). All standard DNA concentrations were determined using PicoGreen (Invitrogen) fluorescence. Standard reaction mixtures contained approximately 10 to 10^6 copies and resulted in lines with slopes of −3.4 to −3.8 (average, −3.6 ± 0.2), corresponding to an average amplification efficiency of 90% ± 6%. The standard DNA for the 16S rRNA gene analysis was genomic DNA from *Escherichia coli* strain EPI300 (Epicentre, Madison, WI). Reactions targeting 16S rRNA genes contained 10^3 to 10^8 copies of standard DNA.

To confirm the specificities of the *Rhodoferax pufM* primer sets, 24 PCR products from representative samples of the entire estuary were cloned and sequenced as described above. The *pufM* genes amplified by the *Rhodoferax*-like *pufM* qPCR primers were on average 88% similar to DelRiverFos06H03 (data not shown). To test the specificity of the gammaproteobacterial *pufM* primer pair, MG1-F/R, 12 PCR products using this primer pair and DNA from the Delaware Bay (9 km from the mouth of the estuary) collected in August 2002 were cloned and sequenced. All tested clones were 92 to 95% similar to *pufM* of HTCC2080 and 94 to 98% similar to the dominant type of marine *pufM* in the Delaware Bay (data not shown). Specificity of the *Rhodobacter* primer set was tested by comparing the sequences of primers RbaF1 and RbaR1 to those of 426 *pufM* sequences from the Delaware estuary, Sargasso, Mediterranean, and Red seas, Monterey Bay, and isolates in the *Rhodobacter*, *Roseobacter*, and *Erythrobacter* genera (see Table S2 in the supplemental material). The best matches to the forward primer (>78% similar with no mismatches at the 3′ end) were *Rhodobacter*-like *pufM* genes from the DNA and RNA libraries from the Delaware Bay and River and the *pufM* genes of the Oregon Coast isolate R2A163 (35), *Rhodobacter blasticus*, and the Southern Ocean *Roseobacter* isolate SO3 (25). The reverse primer matched well to *Rhodobacter*-like *pufM* genes from the DNA and RNA libraries from the Delaware Bay and River and to the *pufM* genes of *Rhodobacter capsulatus*, *Rhodobacter blasticus*, and *Rhodobacter sphaeroides*, as well as to those of *Roseobacter* isolates R2A163 and OCH114, *Roseobacter denitrificans*, *Roseobacter litoralis*, and *Roseobacter* isolate BS90.

Nucleotide sequence accession numbers. The sequences of all *pufM* clones were deposited in GenBank under the accession numbers EU191236 to EU191609.

RESULTS

Diversity and composition of Delaware estuary *pufM* libraries. We constructed three *pufM* libraries from bacterial nucleic acids from the freshwater end of the Delaware estuary and one from the mouth of the bay (Table 2). Protein sequences were grouped at the 97%-similarity levels as recommended for protein-encoding genes (27). The four libraries were composed of 21 to 33 groups, with coverage ranging from 57 to 79%. The Chao I richness estimates for these four libraries were not statistically different from each other (Table 2).

We compared the Delaware River and Bay clones to known *pufM* genes using BLASTX (Table 3). The December river library was dominated by clones (88% of all clones) closely related to *pufM* of DelRiverFos06H03, which is hypothesized to be representative of freshwater AAP *Betaproteobacteria* (37). The August river DNA library also contained *pufM* genes related to the betaproteobacterial DelRiverFos06H03 and other freshwater *pufM* genes from Lake Fryxell. Approximately half of the August river *pufM* genes were related to the *pufM* gene of the cultured gammaproteobacterium *Congregibacter litoralis* KT71 (15), but the average similarity of the amino acid sequences was only 92% (Table 3). The genes similar to *Rhodobacter* and *Rhodobacter*-like *pufM* genes, including that of the hypothesized estuarine type in fosmid clone DelRiverFos13D03 (37), comprised less than 5% of the cloned *pufM* genes in both river DNA libraries (Table 3).

The composition of the river cDNA library was similar to that of its DNA counterpart except that the gammaproteobacterial gene did not dominate (Table 3). Approximately 30% of the *pufM* transcripts in the river sample were closely related to DelRiverFos06H03 *pufM*, with a corresponding average amino acid similarity of 94% (Table 3), and another 20% were similar to Lake Fyxell *pufM* genes. Only 4% of *pufM* in cDNA clones was similar to estuarine DelRiverFos13D03 *pufM*. Genes most similar to those of the *Gammaproteobacteria* comprised approximately 14% of the cDNA library.

In contrast to the river DNA libraries, the August bay DNA library was dominated by genes similar to the *pufM* genes from the Monterey Bay BAC clone EBAC000-29C02 (37% of all clones) and *Congregibacter litoralis* KT71 (32%) (Table 3). The *pufM* genes in Delaware Bay clones similar to the *pufM* gene in the Monterey Bay BAC clone ranged in similarity from 84 to 98%, with an average of 95% (Table 3). Products of the *Congregibacter*-like *pufM* genes were on average 95% similar at the amino acid level. The remaining 27% of the bay library was composed of clones with genes similar to the *pufM* genes from cultured representatives in the alpha-3 and alpha-4 (*Roseobacter* and *Erythrobacter*) subclasses of the *Proteobacteria*. In the bay library, no *pufM* gene was related to that of the estuarine DelRiverFos13D03 fosmid clone, and there were no freshwater representatives in this library.

We further examined the relationships of the Delaware estuary *pufM* genes and transcripts with other *pufM* genes by constructing a phylogenetic tree with dominant members of the Delaware estuary libraries (Fig. 1). The tree contained a cluster of *Rhodoferax*-like freshwater *pufM* sequences, comprised of Delaware River *pufM* genes and transcripts as well as *pufM* sequences from Lake Fryxell (19). The remaining clusters in the tree contained *pufM* genes from cultured and uncultured representatives isolated from marine and coastal environments. One group contained *pufM* genes similar to those of the alpha-3 (*Rhodobacter*- and *Roseobacter*-like) and alpha-4 (*Erythrobacter*-like) subgroups of *Proteobacteria*. The hypothesized estuarine type of *pufM*, DelRiverFos13D03 fosmid clone, and river DNA and cDNA clones fell in this group. The *pufM*

TABLE 3. BLAST analysis of Delaware estuary *pufM*[a]

Library	Top BLASTX hit	Accession no.	Phylogenetic group[b]	% of clones	Avg % identity[c]
December 2001 river	**DelRiverFos06H03**	**AAX48200**	**Beta**	88	99
	Roseateles depolymerans	BAB19668	Beta	4	94
	Lake Fryxell c1	AAO62372	Alpha-1	3	96
	Lake Fryxell c7	AAO62378	Alpha-3	1	100
	Rhodobacter blasticus	**BAA22642**	**Alpha-3**	1	93
	Thiocapsa roseopersicina	CAD66535	Gamma	1	93
	Thiocystis gelatinosa	BAA22650	Gamma	1	90
August 2002 river	*Congregibacter litoralis* KT71	ZP_01104362	Gamma	51	92
	DelRiverFos06H03	**AAX48200**	**Beta**	16	98
	Lake Fryxell c1	AAO62372	Alpha-1	15	93
	Lake Fryxell c8	AAO62379	Alpha-1	8	89
	Blastomonas sp. strain NT12	BAA25728	Alpha-4	7	95
	Rhodobacter blasticus	**BAA22642**	**Alpha-3**	2	95
	DelRiverFos13D03	**AAX48162**	**Alpha-3**	1	98
August 2002 river cDNA	**DelRiverFos06H03**	**AAX48200**	**Beta**	30	94
	Lake Fryxell c1	AAO62372	Alpha-1	19	94
	Blastomonas natatoria	BAA25728	Alpha-4	14	90
	Congregibacter litoralis KT71	ZP_01104362	Gamma	14	92
	Roseiflexus sp. strain RS-1	ZP_01355478	Green non-sulfur	7	72
	DelRiverFos13D03	**AAX48162**	**Alpha-3**	4	98
	Jannaschia sp. strain CCS1	YP_508114	Alpha-3	4	94
	Roseococcus thiosulfatophilus	AAL57746	Alpha-1	3	86
	Porphyrobacter neustonensis	BAA25904	Alpha-4	3	93
	Lake Fryxell c8	AAO62379	Alpha-1	1	92
	Rhodobacter veldkampii	BAC54030	Alpha-3	1	92
August 2002 bay	**EBAC000–29C02**	**AAM48603**	**Gamma**	37	95
	Congregibacter litoralis KT71	ZP_01626201	Gamma	32	95
	Rhodobacter blasticus	**BAA22642**	**Alpha-3**	21	89
	Roseobacter sp. strain SYOP2	AAT79391	Alpha-3	3	98
	Porphyrobacter sanguineus	BAA25723	Alpha-4	3	87
	Jannaschia sp. strain CCS1	YP_508114	Alpha-3	2	95
	Blastomonas sp. strain NT12	BAA77030	Alpha-4	1	90
	Hawaii envhot3	AAL02391	Gamma	1	95

[a] All *pufM* genes were compared to database sequences (January 2007) using BLASTX. Sequences similar to those boldfaced in the table were targeted by qPCR in this study.

[b] Phylogenetic group assignment of each top BLAST hit was based on 16S rRNA for cultured bacteria and on *pufM* for uncultured bacteria. Alpha, beta, and gamma refer to subclasses of the *Proteobacteria*. Lake Fryxell clone 1 and 7 *pufM* phylogenetic affiliations were assigned to the alpha-1 and alpha-3 subclasses of *Proteobacteria* (19). The *pufM* gene in clone EBAC000-29C02 was assigned to the *Gammaproteobacteria* (15).

[c] Average *pufM* product percent amino acid identity to the top BLASTX hit for all clones in each library is noted.

clones from the August 2002 bay and March 2005 turbidity maximum (66 km from the mouth of the bay) samples also clustered with these sequences. The last cluster of *pufM* genes, the Delaware marine group, was composed of genes from Delaware Bay clones and from the hypothesized gammaproteobacterial Monterey Bay bacterial artificial chromosome (BAC) clones, EBAC000-29C02 and -65D09. The Delaware marine group cluster also contained the *pufM* gene from the gammaproteobacterium HTCC2080, isolated from oligotrophic oceanic waters (6), and a *pufM* clone from San Pedro Channel (SPOTS1).

Quantitative mapping of *pufM* ecotypes. To examine the distribution of *pufM* genes along the salinity gradient of the estuary, we designed qPCR primers to target three major groups of *pufM* genes hypothesized to be abundant in the estuary (Table 1). One was the *pufM* gene of the fosmid clone DelRiverFos06H03, thought to be representative of freshwater AAP bacteria (37). The second type, *Rhodobacter*-like, was hypothesized to be ubiquitous throughout the estuary. This ecotype comprised less than 5% of the clones in DNA libraries from either end of the estuary (Table 3), but genes related to *pufM* from DelRiverFos13D03 and other *Rhodobacter*-like *pufM* genes made up approximately 10% of the August river cDNA library. Additionally, since *Rhodobacter* species are associated with particles (12) and a large proportion of estuarine AAP bacteria are associated with particles (38), we hypothesized that *pufM* genes in this clade would be abundant throughout the estuary.

The third ecotype targeted by qPCR, the Delaware marine group, was a dominant *pufM* sequence in the Delaware Bay library, comprising 37% of the clones (Table 3), and was similar to genes from Monterey Bay BAC clones and the gammaproteobacterium HTCC2080 (6). It was distinct, however, from the second-most-abundant gammaproteobacterial *pufM* gene in the Delaware Bay library. This *pufM* clade, comprising 32% of the Delaware Bay clones, was a loose cluster of genes that were on average 95% similar to *pufM* of *Congregibacter litoralis*. This type of *pufM* gene was not targeted by qPCR, since these sequences did not fall in a tight cluster.

Two of the *pufM* types varied consistently with salinity in the

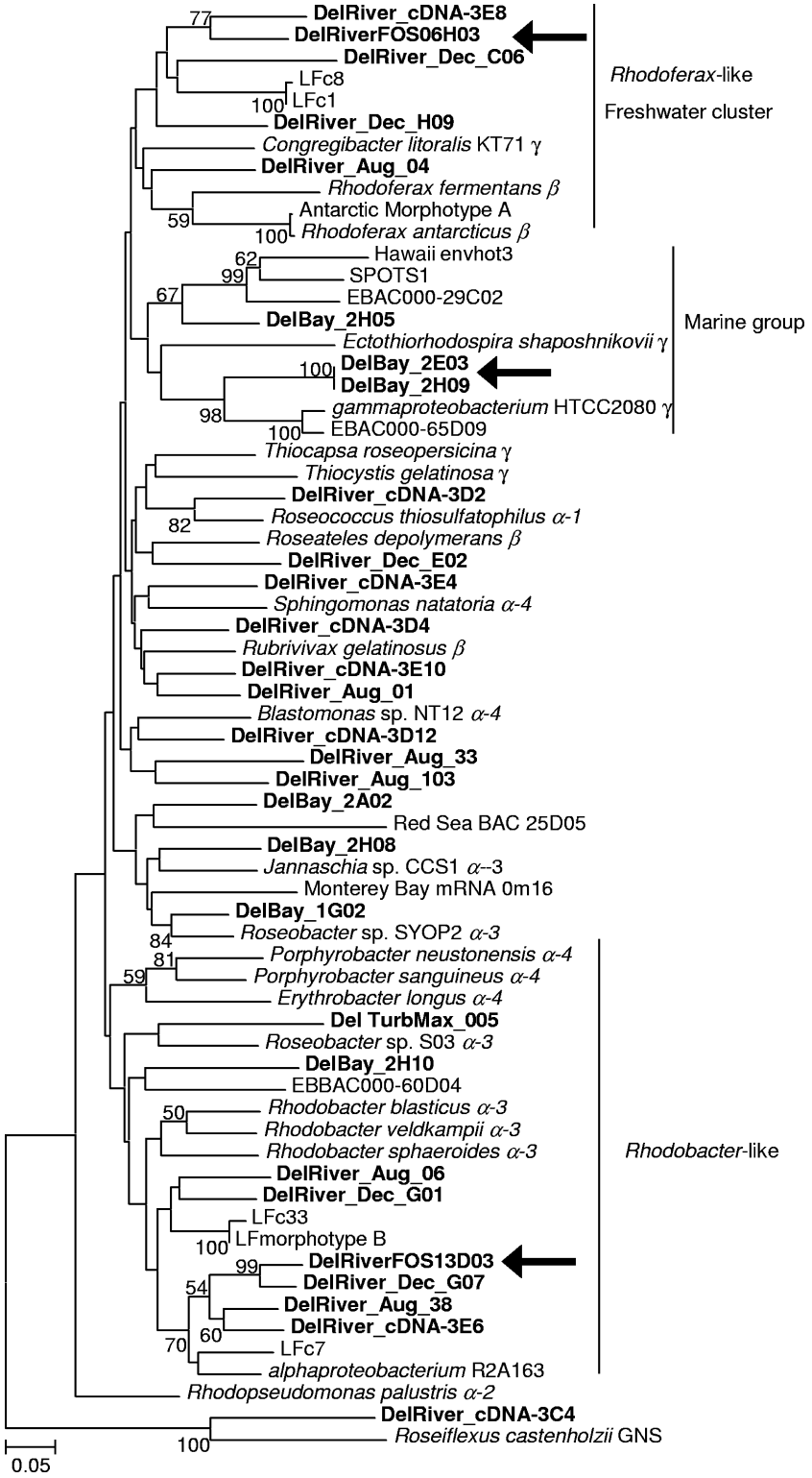

FIG. 1. Relationships of *pufM* genes in the Delaware River and Bay. Delaware clones from the river (DelRiver), turbidity maximum (Del TurbMax) and bay (DelBay) are in bold. Lake Fryxell clones are abbreviated "LF." Alpha-, beta-, and gammaproteobacterial clusters are designated on the basis of the 16S rRNA gene. α-2, α-3, and α-4 refer to subclasses of *Alphaproteobacteria*. Delaware cDNA clones are indicated (cDNA). Fosmid clones described previously (38) are marked DelRiverFOS. Ecotypes targeted by qPCR are indicated by arrows (100% match of primers to fosmid or plasmid sequences). The scale bar represents 5 nucleotide substitutions per 100 positions. *Chloroflexus aurantiacus* was the outgroup.

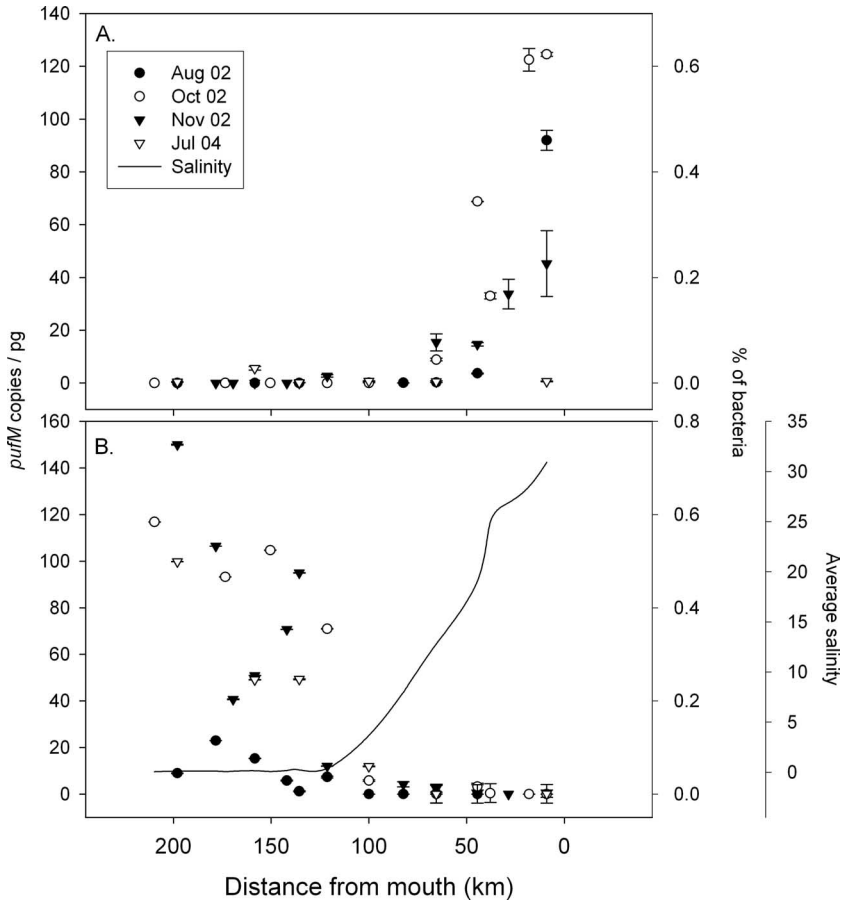

FIG. 2. Distribution of two *pufM* ecotypes normalized to pg of total DNA in the Delaware estuary. Ecotypes targeted by qPCR were Delaware marine group (gammaproteobacterial) (A) or *Rhodoferax*-like (betaproteobacterial) (B) *pufM* genes. Error bars show the standard errors for four qPCRs. The average salinity for all four transects is plotted in panel B. The percentage of bacteria was calculated assuming 2 fg DNA per bacterial cell.

Delaware estuary (Fig. 2). The Delaware marine group was restricted to waters with a salinity of >11 (Fig. 2A). The abundance of this *pufM* gene covaried significantly with salinity ($r = 0.57$; $P < 0.001$; $n = 49$) and was inversely correlated to nitrate concentrations ($r = -0.69$; $P < 0.001$; $n = 27$). At the mouth of the bay in 2002, this gene ranged from 50 to 125 copies/pg of total bacterial DNA, corresponding to 0.25 to 0.63% of bacteria, assuming 2 fg DNA per bacterial cell. This *pufM* gene was not detected at significant levels in July 2004. In contrast, the *Rhodoferax*-like *pufM* ecotype was restricted to waters with a salinity of <5, and its abundance was inversely correlated with salinity ($r = -0.65$; $P < 0.001$; $n = 49$) and chlorophyll ($r = -0.41$; $P < 0.01$; $n = 39$) regardless of the season (Fig. 2B). Additionally, this freshwater *pufM* sequence positively correlated with nitrate ($r = 0.51$; $P < 0.01$; $n = 27$) and phosphate concentrations ($r = 0.58$; $P < 0.01$; $n = 27$). In three transects, maximum abundances of *Rhodoferax*-like *pufM* were similar to those of the marine group, ranging from 40 to 150 copies/pg, which corresponds to 0.2 to 0.75% of the total bacterial community. In August 2002, this type was not abundant even in the least-saline stations, reaching only 20 copies/pg (Fig. 2B). The hypothesized estuarine *Rhodobacter*-like *pufM* gene was ubiquitous in the estuary but did not vary consistently through the salinity gradient (Fig. 3). This *pufM* gene was more abundant in the lower-salinity waters of the estuary (125 km to 200 km from the mouth) during October and November 2002 (Fig. 3A), whereas in August 2002 and July 2004, the *Rhodobacter*-like *pufM* gene was more abundant in the higher-salinity stations (Fig. 3B). In the riverine section of the estuary, *Rhodobacter*-like *pufM* genes were not as abundant as *Rhodoferax*-like *pufM*, reaching only 120 copies/pg DNA or about 0.6% of all bacteria (Fig. 3A). The abundance was not significantly correlated with nutrient concentrations ($r < 0.31$; $P > 0.05$; $n = 27$) or chlorophyll ($r = 0.12$; $P > 0.05$; $n = 38$).

We estimated the contribution of these three ecotypes to the bacterial community as a whole and to the AAP bacterial community. In the entire data set, the sum of the three types of AAP bacteria comprised up to 1.6% of bacteria, with an average of 0.14% (calculated from data in Fig. 2 and 3), assuming one copy of *pufM* per genome and two 16S rRNA genes or 2 fg DNA per bacterial cell (14, 28). These estimates were also compared to the total AAP bacterial abundance estimated previously (38) by *pufM* qPCR (Table 4). In five cruises, the three types of *pufM* comprised on average 5.7% of all bacteria containing the *pufM* gene (Table 4). In the middle portions of the estuary, the contribution of the three *pufM* types to the

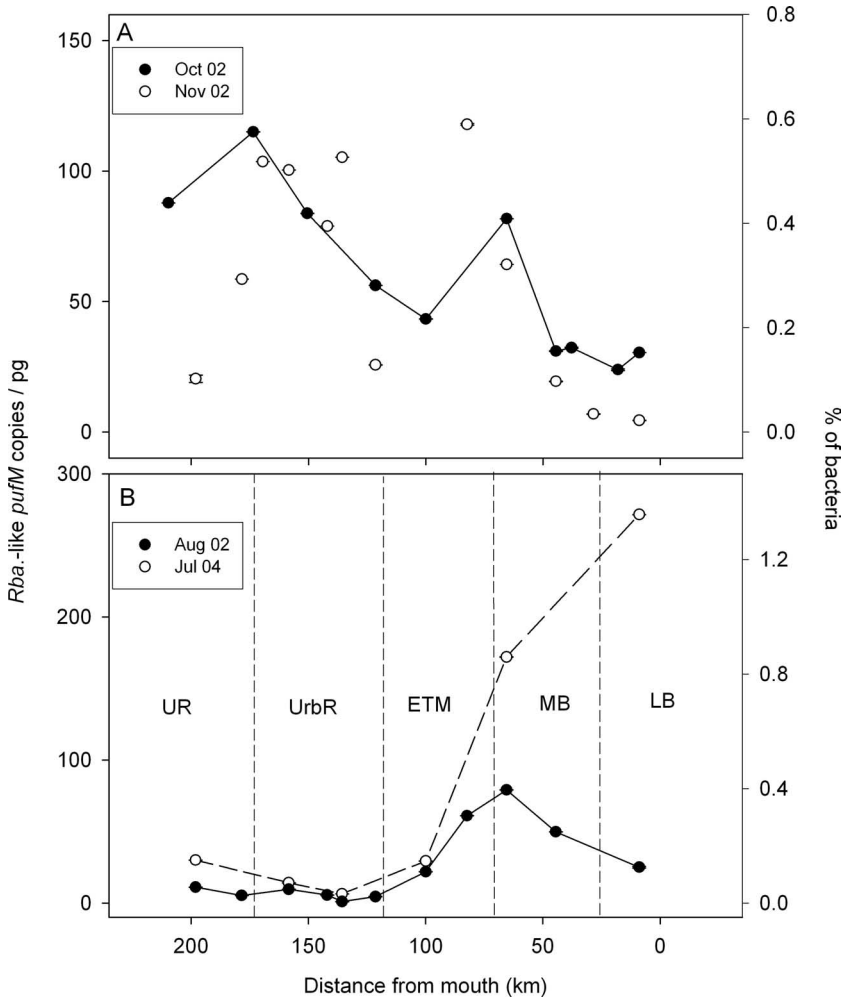

FIG. 3. Distribution of *Rhodobacter*-like *pufM* ecotypes, normalized to pg of total DNA, in the Delaware estuary in autumn (A) or summer (B). Error bars, each based on the standard error for four qPCRs, are smaller than the symbols. Dashed vertical lines in panel B delineate the five regions of the estuary: LB, lower bay (0 to 25 km); MB, midbay (25 to 70 km); ETM, turbidity maximum (70 to 115 km); UrbR, urban river (115 to 175 km); UR, upper river (175 to 215 km). The percentage of bacteria was calculated assuming 2 fg DNA per bacterial cell.

total bacterial and AAP bacteria community averaged about 0.08% and 4.3%, respectively. In the lower bay and upper river, the three types of AAP bacteria examined in this study were more abundant and comprised 12 and 8% of the AAP bacterial community, respectively (Table 4).

Particle-associated *pufM* genes. To determine if specific groups of AAP bacteria were associated with particles, we estimated abundances of particle-attached *pufM*-containing bacteria by subtracting values in the free-living fraction from those of the corresponding whole water during a transect in

TABLE 4. Contributions of three ecotypes to total AAP bacterial communities in the Delaware estuary[a]

Region[b] (n[c])	% of bacteria (±SE) containing *pufM*			
	Rhodobacter-like	*Rhodoferax*-like	Marine group	Sum
Lower bay (7)	3.1 (0.15)	0.0049 (0.006)	8.8 (0.21)	12.0 (4.6)
Midbay (15)	3.9 (0.11)	0.044 (0.25)	2.0 (0.13)	6.0 (1.7)
Turb. max. (9)	2.8 (0.20)	0.19 (0.037)	0.017 (0.0018)	3.1 (1.2)
Urban river (18)	1.9 (0.06)	1.5 (0.11)	0.0047 (0.0067)	3.5 (0.72)
Upper river (6)	2.8 (0.031)	5.0 (0.058)	0.00010 (0.000014)	7.8 (2.6)
Entire estuary (55)	2.9 (0.55)	1.1 (0.79)	1.7 (2.9)	5.7 (0.90)

[a] The *Rhodobacter*-like, *Rhodoferax*-like, and marine group (gammaproteobacterial) *pufM* genes were compared to the total AAP bacterial community by normalizing to total *pufM* abundance (36). "Sum" is the total of the three percentages.

[b] Regions were defined as in reference 37 by distance from the mouth of the bay: lower bay (0 to 25 km), Midbay (25 to 70 km), turbidity maximum (Turb. max.) (70 to 115 km), urban river (115 to 175 km), or upper river (175 to 215 km).

[c] n, number of samples in each region from five cruises (August, October, and November 2002, July 2004, and March 2005).

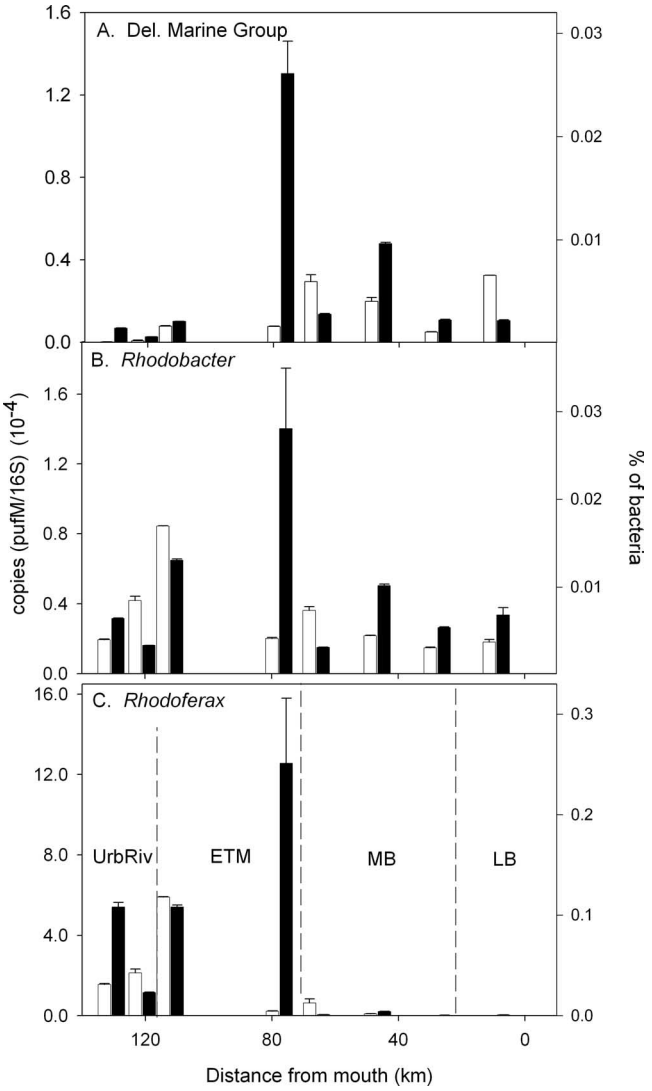

FIG. 4. Distribution of three *pufM* ecotypes in whole water and the free-living bacterial fraction in March 2005. Abundance of Delaware marine group (A), *Rhodobacter*-like (B), or *Rhodoferax*-like (C) *pufM* genes was normalized to 16S rRNA gene abundance. The percentage of bacteria was calculated assuming two 16S rRNA gene copies per bacterial cell.

2005 (Fig. 4). The variation of the three *pufM* types in the total community throughout the estuary in 2005 was similar to that for *pufM* in the free-living fraction in 2002 and 2004 (Fig. 2 and 3). However, in contrast to previous transects, *Rhodoferax*-like *pufM* was more abundant than the other two ecotypes. The estimated fraction of bacteria comprising the three *pufM* ecotypes was 10-fold less in 2005 than in 2002 to 2004, in part due to how the bacteria were collected (whole water or GF/D filtration versus 0.8-μm polycarbonate filtration). About 80 km from the mouth of the bay (Fig. 4), particle-associated AAP bacteria were a very large fraction of the total, probably because of high particle concentrations (38). But overall, the average (± standard error) percentages of particle-associated *pufM* genes were 22% ± 36%, 12% ± 31%, and 24% ± 38% for the Delaware marine group, *Rhodobacter*-like, and *Rhodoferax*-like types, respectively (calculated from data in Fig. 4). These percentages do not differ significantly (analysis of variance, $P > 0.05$) and are also not statistically different from 39% ± 18%, the average percentage of total AAP bacteria associated with particles in the Delaware Estuary (38).

DISCUSSION

The goal of this study was to examine relationships among estuarine AAP bacteria and to determine if ecotypes inferred from phylogenetic analyses varied systematically within the salinity gradient of the estuary. We hypothesized that the distribution of AAP bacterial types would follow the patterns of bacterial groups previously determined using the 16S rRNA gene. Groups of freshwater, brackish, and marine ecotypes of *pufM* sequences were determined by phylogenetic analyses, and these patterns were confirmed by qPCR abundance estimates through the estuary. In contrast to our initial hypothesis, the distribution of the three types of *pufM* genes did not coincide entirely with the patterns of *Alpha*-, *Beta*-, and *Gammaproteobacteria* typically observed in studies of rRNA genes in estuaries.

Salinity probably influenced the distribution of the betaproteobacterial *Rhodoferax*-like AAP bacteria in the estuary. The hypothesized freshwater *pufM* genes clustered with *pufM* genes from organisms originally isolated from low-salinity environments, such as *Rhodoferax* and *Roseateles*, and from uncultured bacteria from the Delaware River and Lake Fryxell. In this study, *Rhodoferax*-like *pufM* genes were restricted to waters with a salinity of <5. These data are supported by the observation that *Rhodoferax*-like *pufM* genes are restricted to estuarine or freshwaters in the Global Ocean Sampling data set (41). The distribution of this group of *pufM* genes is consistent with the distribution of *Betaproteobacteria* in the Delaware and other estuaries (5, 7, 11). This restriction to low-salinity waters, however, may be in part explained by the inverse relationship between nutrient concentrations and salinity in the Delaware ($r = -0.75$; $P < 0.001$; $n = 27$) and other estuaries (39).

Unexpectedly, our qPCR results indicated that the abundance of the gammaproteobacterial AAP bacteria covaried with salinity. Studies using 16S rRNA gene clone libraries or fluorescence in situ hybridization indicate that the *Gammaproteobacteria* do not vary systematically with salinity (7, 10). The distribution observed in this study may be related to the trophic status of these waters (as indicated by nitrate concentrations), not just salinity. In the Delaware estuary, the positive relationship between gammaproteobacterial AAP bacteria and salinity may be due in fact to nitrate, because there is an inverse relationship between nitrate concentrations and salinity in the estuary ($r = -0.75$; $P < 0.001$; $n = 27$).

Additionally, different groups of gammaproteobacterial AAP bacteria may be adapted to different trophic conditions. AAP bacteria in the OM60 clade of *Gammaproteobacteria* appear to be comprised of two groups based on individual gene phylogeny and synteny, and these groups occupy distinct habitats (41). In the Delaware estuary, there were two types of gammaproteobacterial *pufM* genes, which are similar to *pufM* genes in other estuarine and coastal communities. The first type, the most abundant one in the Delaware Bay library, is similar to the *pufM* gene from the gammaproteobacterium

HTCC2080 (6) and the Monterey Bay BAC clone EBAC000-65D09 (4) (Fig. 1). This type accounts for 37% of the *pufM* genes amplified by the Delaware marine group qPCR primers (Table 3). The second type, related to *Congregibacter litoralis* (15), comprised a large portion of the *pufM* genes and mRNA transcripts from the Delaware River and approximately one-third of *pufM* clones from the Delaware Bay. Unfortunately, the average percent similarity of Delaware estuary *pufM* genes to the *C. litoralis pufM* gene was low (Table 3), resulting in this cluster being too loose to be examined by a single qPCR assay.

The qPCR data indicated that the *Rhodobacter*-like ecotype covaried with salinity and that there was a seasonal influence on this relationship. This seasonal difference is not explained by nitrate, since nitrate concentrations are always highest in the urban river and at the turbidity maximum of the estuary, regardless of season (29). Instead, it may be partly explained by the broad range of *Rhodobacter*- and *Roseobacter*-like AAP genes amplified by this primer set. The nucleotide sequences of the forward and reverse Rba qPCR primers match, on average, 64 and 86%, respectively, to nucleotide sequences of representative *Rhodobacter* and *Roseobacter pufM* genes (see Table S2 in the supplemental material). Subgroups of bacteria in the *Rhodobacter* group, particularly those related to *Sagittula stellata* and *Ruegeria* spp., vary systematically with season and inorganic nitrogen and particulate organic matter concentrations in a salt marsh creek (12), although *pufM* genes have not been found in cultured representatives of these bacterial subgroups. More data on AAP bacteria in the *Rhodobacter* clade are needed.

Other environmental factors probably influence the survival of specific groups of AAP bacteria in the estuary. Light attenuation in the turbidity maximum of the Delaware estuary is high, and low light availability may negatively influence some AAP bacteria. This, along with increasing allochthonous nutrient loads, may in part explain the low abundance of the Delaware marine group in the Delaware River. This group may be representative of AAP bacteria that are adapted to more oligotrophic, clearer waters, such as those of coastal areas and open oceans (6, 15). Additionally, top-down factors, such as grazing, could also preferentially remove certain ecotypes, such as the betaproteobacterial *Rhodoferax*, from the midestuary. Some *Betaproteobacteria* are fast-growing, large cells which are controlled by grazing pressure (32). Since the cell size of AAP bacteria can be large in coastal and oceanic environments (8, 31), preferential grazing on larger cells (30) may help to explain the abundance of certain types of AAP bacteria.

Detrital particle concentrations also appear to be important in affecting the distribution of AAP bacteria, since our qPCR results indicated that a large fraction of all three types of AAP bacteria was associated with particles. The association of AAP bacteria with particles may be due to oxygen availability (38). In culture, *Congregibacter litoralis* forms aggregates and grows optimally in semiaerobic conditions (15). Additionally, the betaproteobacterial isolate *Roseateles depolymerans* increases production of bacteriochlorophyll *a* and reaction center proteins under microaerobic conditions (0.2 to 2% oxygen) (33). The particle attachment preference of total AAP bacteria (38), as well as the findings in this study, suggest that association with particles is common among all AAP bacteria, particularly in coastal and estuarine environments. Particle attachment by AAP bacteria may even be common in the open ocean, since in the Sargasso Sea, the estimated AAP abundance was twofold lower in free-living bacterioplankton (<0.8 μm) than in the 3- to 20-μm fraction (41).

The qPCR assays targeted three *pufM* types hypothesized to be ecologically interesting based on the clone library results and a previous fosmid library study of AAP bacteria (37). In total, the three groups examined by qPCR comprised on average only 5.7% of all *pufM* genes in the estuary, much lower than what we expected based on the clone library results. This difference may reflect the well-known problems with trying to use clone library results for quantitative analyses, and it also suggests a higher diversity of AAP bacteria than what was captured in the four PCR libraries constructed for this study.

This study was the first to examine how specific types of AAP bacteria vary with respect to environmental parameters such as salinity and nutrients. The distribution of the betaproteobacterial *pufM* types within the estuary as determined by qPCR was consistent with what we expected based on its phylogenetic affiliation. However, the estuarine distribution of the alpha-3-proteobacterial *Rhodobacter*-like and gammaproteobacterial *pufM* genes did not coincide with what had previously been observed in studies using the 16S rRNA gene. Further investigations into the distribution and activity of these and other types of AAP bacteria, such as those containing the gammaproteobacterial *pufM* genes, may provide further clues to potentially diverse ecological adaptations by AAP bacteria.

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