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Polyphyletic photosynthetic reaction centre genes in oligotrophic marine *Gammaproteobacteria*

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Summary

Ecological studies indicate that aerobic anoxygenic phototrophic bacteria (AAP) that use bacteriochlorophyll to support phototrophic electron transport are widely distributed in the oceans. All cultivated marine AAP are alpha-3 and alpha-4 *Proteobacteria*, but metagenomic evidence indicates that uncultured AAP *Gammaproteobacteria* are important members of ocean surface microbial communities. Here we report the description of obligately oligotrophic, marine *Gammaproteobacteria* that have genes for aerobic anoxygenic photosynthesis. Three strains belonging to the OM60 clade were isolated in autoclaved seawater media. Polymerase chain reaction assays for the *puf*M gene show that these strains contain photosynthetic reaction centre genes. DNA sequencing and phylogenetic analysis indicate that the *puf*M genes are polyphyletic, suggesting multiple instances of lateral gene transfer. Peptide sequences from six photosynthesis genes (*puf*L, *puf*M, *puf*C, *puf*B, *puf*A and *puh*A) were detected by proteomic analyses of strain HTCC2080 cells grown aerobically in seawater. They closely match predicted peptides from an environmental seawater bacterial artificial chromosome clone of gammaproteobacterial origin, thus identifying the OM60 clade as a significant source of gammaproteobacterial AAP genes in marine systems. The cell yield and rate of growth of HTCC2080 in autoclaved, aerobic seawater increased in the light. These findings identify the OM60 clade as a source of *Gammaproteobacteria* AAP genes in coastal oceans, and demonstrate that aerobic, anoxygenic photosynthetic metabolism can enhance the productivity of marine oligotrophic bacteria that also grow heterotrophically in darkness.

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Introduction

Aerobic anoxygenic phototrophic bacteria (AAP) (Yurkov and Beatty, 1998) that use bacteriochlorophyll to support phototrophic electron transport were recently reported to be widely distributed in the oceans (Kolber *et al*., 2000; 2001). Since the time of that discovery (Kolber *et al*., 2000), AAP strains belonging to the alpha-3 and alpha-4 *Proteobacteria* have been isolated from diverse habitats. These strains grow optimally in organic rich-media containing peptone and yeast extract (Allgaier *et al*., 2003; Koblizek *et al*., 2003). However, metagenomic evidence indicates that uncultured AAP *Gammaproteobacteria* are also important components of marine bacterioplankton (Beja *et al*., 2002).

The isolates we describe in this paper are members of the OM60 clade of marine bacteria. This clade was first identified by phylogenetic analysis of environmental 16S rRNA clones from coastal seawater samples (Rappé *et al*., 1997). The first isolate from this clade, KT71, was described in 2001 (Eilers *et al*., 2001). More reports of isolated strains from this clade followed (Qian and Wilkinson, 1991; Connon and Giovannoni, 2002; Brinkmeyer *et al*., 2003; Cho and Giovannoni, 2004; Agogue *et al*., 2005), as GenBank entries for environmental clones from the OM60 clade accumulated, attesting to its significance. Supporting this conclusion, studies employing rRNA probes to detect the OM60 clade by fluorescence *in situ* hybridization (FISH) in samples from the North Sea revealed cell abundances ranging from 6% to 11% of total bacterial counts.

Among the isolates described above are a number that emerged when our laboratory began to apply culture isolation methods based on dilution in natural seawater media (Button *et al*., 1993; Connon and Giovannoni, 2002). We found that members of the OM60 clade readily reproduce on autoclaved seawater. Experimenting with liquid chromatography-electrospray ionization tandem

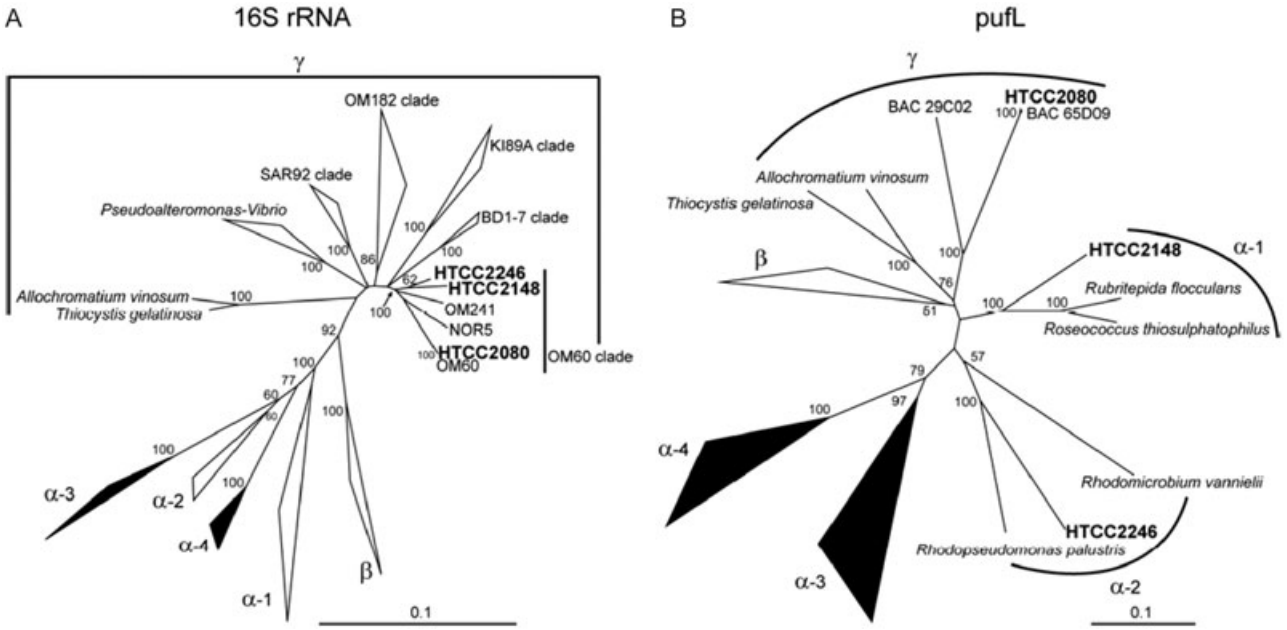

Fig. 1. Phylogenetic relationships of 16S rRNA (A) and *puf*L gene (B) sequences for the gammaproteobacterial AAP isolates and other proteobacteria. A total of 989 and 703 unambiguously aligned nucleotide positions for the 16S rRNA gene (A) and *puf*L gene (B), respectively, were used for neighbour-joining analyses. Names in bold indicate the three AAP isolates. Brackets indicate the boundary of each phylogenetic group, and black triangles represent alpha-3 and alpha-4 proteobacteria, to which previously cultured marine AAP belong. Bootstrap proportions over 50% from the neighbour-joining analysis are shown. Scale bar, 0.1 substitutions per nucleotide position.

mass spectrometry (LC-ESI-MS/MS) methods, we were surprised to find compelling matches between peptides from isolate HTCC2080 and environmental bacterial artificial chromosome (BAC) clones reported by Beja and colleagues (2002). Here we report the results of proteome measurements and gene sequencing that indicate a complex evolutionary origin for AAP in the OM60 clade. We also report environmental data that suggest that OM60 clade is most abundant in coastal surface waters, and physiological studies that demonstrate enhanced growth of strain OM60 in the light. These findings suggest that AAP bacteria of the OM60 clade are significant contributors to coastal ocean ecosystems.

Results and discussion

The ocean surface is a highly oligotrophic habitat, leading us to hypothesize that uncultured, phylogenetically distinct, oligotrophic cells might be the prevalent AAP species in the oceanic surface. Screening of the High Throughput Culture Collection (HTCC), a microbial culture collection maintained at Oregon State University, revealed *puf* genes among several oligotrophic strains that had been isolated by dilution-to-extinction in natural seawater-based media (Connon and Giovannoni, 2002; Rappe *et al*., 2002; Cho and Giovannoni, 2004).

In screens using primers for amplifying photosynthetic reaction centre genes (*puf*L and *puf*M), three *puf*LM-containing strains were identified from approximately 250 isolates cultured from coastal seawater of the Pacific Ocean. In ribosomal RNA gene trees, these *puf*LM-containing strains (HTCC2080, HTCC2148 and HTCC2246) and several environmental clones formed a distinct monophyletic group within the OM60 clade (Cho and Giovannoni, 2004) of the larger OMG group (Fig. 1A). These strains are the first cultured marine *Gammaproteobacteria* isolates bearing photosynthetic gene clusters. However, *puf*L, *puf*M and *bch*X gene trees revealed polyphyletic origins for photosynthesis genes from these isolates (Fig. 1B; Fig. S1). The *puf*L and *puf*M phylogenetic trees, strongly supported by bootstrap frequencies, showed that the *puf* genes of strain HTCC2080 are affiliated with the *Gammaproteobacteria*, while the HTCC2148 and HTCC2246 genes were closest to homologues from the alpha-1 and alpha-2 *Proteobacteria* respectively. These robust but incongruent trees are evidence of horizontal gene transfer of the photosynthetic gene clusters.

The alpha-1 and alpha-2 proteobacterial *puf* gene types of strains HTCC2148 and HTCC2246, respectively, have never before been observed in marine environments, even in environmental gene cloning studies. The *Gammaproteobacteria puf* genes found in strain HTCC2080 have been observed only in a coastal surface seawater BAC clone library (Beja *et al*., 2000a; 2002). The *puf*M and *puf*L gene sequences of HTCC2080 were 99% similar

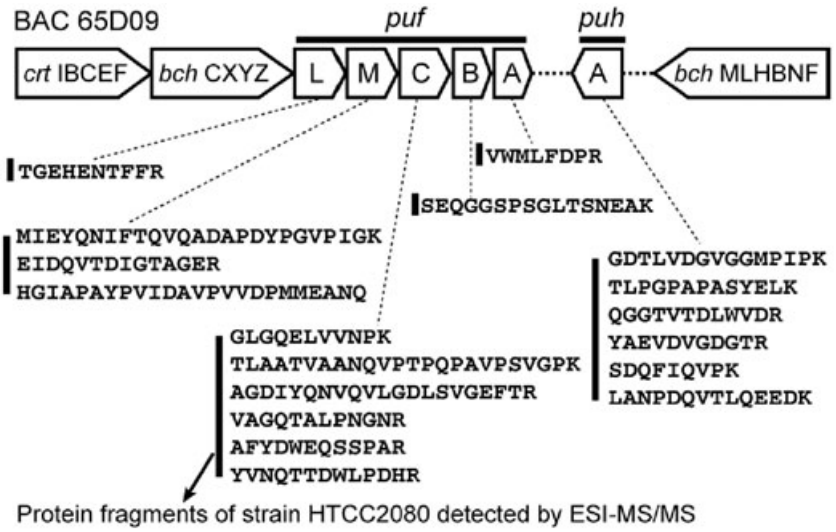

Fig. 2. Protein fragments of photosynthetic genes of strain HTCC2080 detected by ESI-MS/MS. The photosynthetic gene arrangement of BAC clone 65D09 (ref. 4) is presented to indicate the genes corresponding to protein fragments detected by mass spectrometry. Brackets indicate amino acid sequences of protein fragments detected from strain HTCC2080.

to the sequences of a surface-seawater BAC clone, 65D09. Translated *puf*M amino acid sequences of HTCC2080 (223 amino acids) were 100% identical to those of BAC clone 65D09. *puf*L gene showed just one amino acid residue difference, from Ile-62 of BAC clone 65D09 to Val of strain HTCC2080. Bacterial artificial chromosome clone 65D09 (89 kb) did not contain a 16S rRNA gene, which made its phylogenetic position uncertain. Beja and colleagues showed that the photosynthetic operon organization of BAC clone 65D09 resembles betaproteobacterial *puf* operons, and inferred that the strain harbouring the photosynthetic operon might be a gammaproteobacterial origin, from the phylogeny of surroundings genes (Beja *et al*., 2002). Accordingly, they designated BAC clone 65D09 as 'beta or gamma proteobacterial origin'. However, BAC clone 65D09 was probably derived from a marine organism similar to strain HTCC2080, demonstrating that sequences derived from the cultivation of novel bacteria can be linked to unidentified contigs from metagenomic libraries.

Proteomic analysis using ESI-MS/MS (Fenn *et al*., 1989) was employed to determine whether the *Gammaproteobacteria* AAP isolates expressed the *puf* genes. Strain HTCC2080 grown in seawater-based oligotrophic medium, LNHM (low nutrient heterotrophic medium) (Connon and Giovannoni, 2002), was chosen as a representative strain because full sequences of the photosynthetic operon of BAC clone 65D09 were available. Initially, we did not detect any phototrophy-related protein fragments from the tryptic digests of the cell lysates. As the AAP photosynthetic apparatus is a membrane-bound protein complex, membrane material was separated from the cell lysates, solubilized and analysed using mass spectrometry. A total of 18 oligopeptide fragments corresponding to *puf*L, *puf*M, *puf*C, *puf*B, *puf*A and *puh*A genes were detected in the cell membranes of a light-grown strain HTCC2080 by ESI-MS/MS (Fig. 2). Even the small, light-harvesting protein α chain (60 amino acids, *puf*A) and β chain (44 amino acids, *puf*B) produced observed peptide fragments. Based on the *puf* operon arrangement of BAC clone 65D09, BAC clone 65D09 contains six photosynthetic reaction centre and light harvesting complex-coding genes (Beja *et al*., 2002). We detected peptide fragments derived from all the genes of this operon, including the photosynthetic reaction centre protein (L, M, H subunits and cytochrome *c*) and the light harvesting protein (α and β chain). All protein fragments detected in strain HTCC2080 were 100% identical to the translated peptide sequences of BAC clone 65D09. These proteomic results show that novel marine aerobic phototrophic bacteria are actively expressing photosynthetic genes when incubated in oligotrophic seawater in the light.

The response of strain HTCC2080 to dissolved organic carbon (DOC) concentrations was typical of an obligate oligotroph (Schut F, 1997), whether incubated in a 14/10 h diurnal light cycle or complete darkness (Fig. 3). In darkness, supplementing 3.0 mM DOC, as a defined mixture of organic carbon compounds (Rappe *et al*., 2002) into LNHM accelerated cellular growth, 14.7 mM DOC reduced specific growth rate and prolonged the lag period, and 29.2 mM DOC stopped cellular growth completely. Upon the first isolation, our AAP isolates did not form colonies in oligotrophic media, such as 1/10 R2A-seawater agar, and could not grow on Marine Agar 2216 and R2A-seawater agar, even after prolonged laboratory adaptation (Cho and Giovannoni, 2004). While the cultures grown in LNHM supplemented with 3.0 mM DOC exhibited no difference between the light and dark conditions, cultures grown in LNHM with no addition of DOC under the light/dark cycle had higher specific growth rates and maximum cell densities than cultures grown in the complete darkness (ANOVA: $P = 0.00347$, Student's *t*-test:

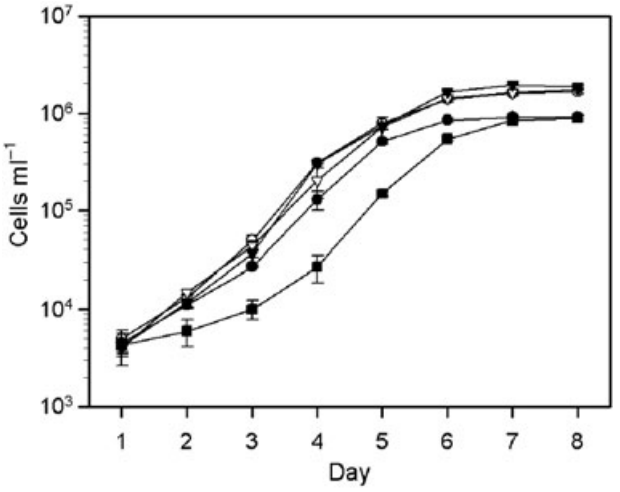

Fig. 3. Growth characteristics of strain HTCC2080 in an LNHM-based medium with 80 μM (○, ●), 3.0 mM (▽, ▼) and 14.7 mM (■) of DOC concentrations, on a diurnal light/dark cycle (open symbols) or in complete darkness (closed symbols). Error bars show standard deviation in triplicate analyses.

$P = 0.013926$; Fig. 3), implying that light energy enhances cellular growth of AAP in oligotrophic oceanic conditions. Functionally, AAP are called photoheterotrophs because they utilize light as an energy source for generating ATP, but require organic material for their cellular growth. This definition of AAP led to a hypothesis that AAP are heterotrophic in organic-rich marine environments and are partly phototrophic in organic-poor oligotrophic environments (Kolber *et al*., 2001; Karl, 2002). Our results are in good agreement with this hypothesis. The carbon content of basal seawater medium (80 μM) used in this study is typical *in situ* DOC concentrations in the open ocean. Therefore, the observation of enhanced cellular growth due to light-driven energy production by expression of the photosynthetic operon (Fig. 2) shows that this system may be advantageous in oligotrophic environments where organic carbon resources are present at very low levels.

Fluorescence *in situ* hybridization with oligonucleotide probes for the OM60 clade shows that these cells are 3.4% ± 1.1% ($n = 11$) of the microbial community in the euphotic zone along the Newport Hydroline (44°39.1′N) in the Pacific Ocean (Fig. 4). Cells hybridizing to the OM60 probe are cocci or short rods of less than 1 μm (Fig. 5). They were found only in the euphotic zone, and generally showed relatively high abundance at deep chlorophyll maxima. The prevalence of AAP and their contribution to ocean ecology (Kolber *et al*., 2000; 2001; Schwalbach MS, 2005; Cottrell *et al*., 2006) are debated. Infrared fast repetition rate fluorometry, which measures bacteriochlorophyll fluorescence, indicated that they contribute 2–5% of phototrophic electron transport in the ocean surface (Kolber *et al*., 2000). Other studies suggest a wide range in the bacteriochlorophyll content of seawater (Sieracki ME, 2006). Microscopic infra-red imaging and quantitative polymerase chain reaction (PCR) have produced estimates that between 2% and 16% of marine bacteria have bacteriochlorophyll (Schwalbach MS, 2005; Cottrell *et al*., 2006).

A draft assembly of the HTCC2080 genome contains 3.58 Mbp (52% GC) and encodes approximately 3259 genes. Preliminary analysis confirms the presence of the photosynthetic reaction centre genes. Consistent with earlier results, the structure of the HTCC2080 *puf* operon is identical to BAC 65D09 and includes enzymes of the bacteriochlorophyll and carotenoid biosynthesis

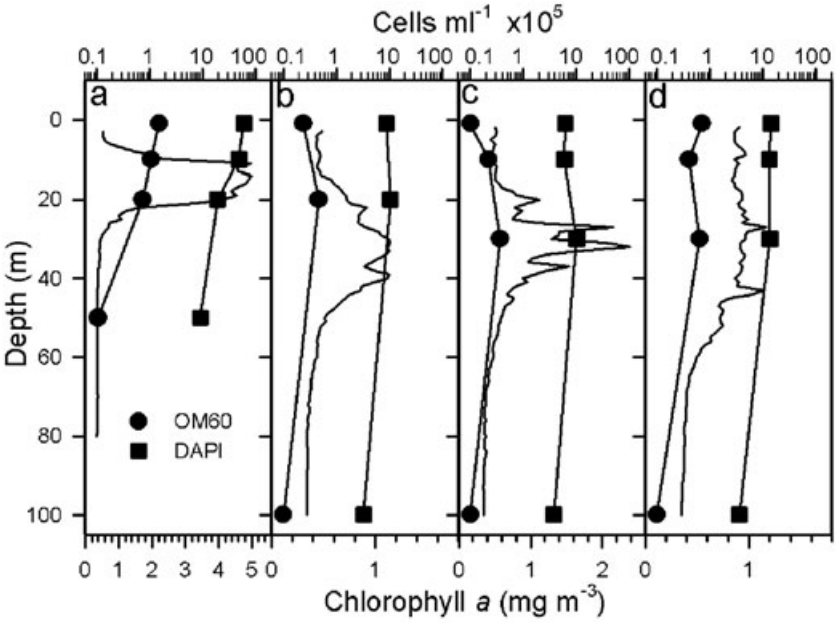

Fig. 4. Cellular abundance of the OM60 clade (●) and total microbial community (■) along the NH hydroline (44°39.1′N) in the Pacific Ocean.
A. NH15 (124°25′W).
B. NH35 (124°53′W).
C. NH65 (125°36′W).
D. NH127 (127°W).
Chlorophyll *a* (lines without symbols) data were taken from CTD measurements.

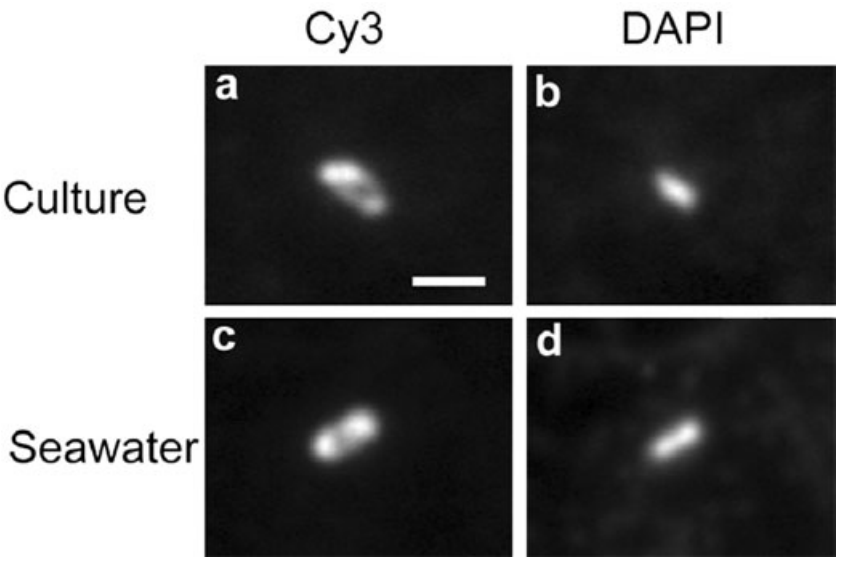

Fig. 5. Microscopic images of cells in the OM60 clade by FISH (A, C) and DAPI staining (B, D). Strain HTCC2080 cells in the exponential phase (A, B) were used as positive controls for FISH. FISH (C) and DAPI (D) images of a seawater sample collected at 30 m depth of station NH65 are shown. The identical fields of view in the DAPI- and Cy3-stained images are shown. Scale bar, 1 μm.

pathways. Consistent with observations from other AAP bacteria (Yurkov and Beatty, 1998), the genome lacks an identifiable ribulosebisphosphate carboxylase, indicating that HTCC2080 cannot grow autotrophically. However, due to the presence of at least one gene encoding phosphoenolpyruvate carboxylase, a low level of CO_2 fixation may be possible. Genes encoding complete glycolysis, pentose phosphate, and amino acid biosynthesis pathways and the TCA cycle can be identified, as well as the glyoxylate shunt enzymes isocitrate lyase and malate synthase. Sulfur metabolism appears similar to other gammaproteobacteria, with the notable presence of a potential sulfite oxidase (SO). Sulfite oxidase is known to occur in only a few other gammaproteobacteria. However, SO appears to be a shared characteristic of almost all phototrophic bacteria examined to date (Yurkov and Beatty, 1998). Finally, the HTCC2080 genome encodes genes for motility, chemotaxis, and a remarkable number of Ton receptors, an active transport system that is directly coupled to transmembrane electrochemical potentials.

We postulate that aerobic anoxygenic photosynthesis contributes to the success of the OM60 clade on continental shelves by providing a system for the generation of cellular electrochemical potential that is not reliant on the availability of metabolically labile organic carbon for respiration. Our results show that members of the OM60 clade are adapted to oligotrophy, express photosynthetic reaction centre genes while growing aerobically as suspended cells in autoclaved seawater, and obtain some growth advantages from their phototrophic capability. Anoxygenic bacterioplankton phototrophy in the ocean is potentially a factor in the trophic efficiency of the microbial loop, and may help explain the very high standing biomass found in the microbial fraction of plankton community. Proteorhodopsin phototrophy (Beja *et al*., 2000b), an analogous anoxygenic phototrophy system, has been found ubiquitously in marine environments among diverse phylogenetic groups, such as the SAR86 gene cluster (Beja *et al*., 2000b; Sabehi *et al*., 2004), uncultured *Alphaproteobacteria (* de la Torre *et al*., 2003), and even the *Bacteroidetes* (Venter *et al*., 2004). Aerobic anoxygenic phototrophic bacteria and proteorhodopsin-bearing bacterioplankton may transform sunlight into biochemical energy to optimize the conversion of limited organic carbon resources into biomass. This study integrates data from environmental genome sequencing with molecular taxonomy and physiological observations. The results reveal unexpected complexity in the evolution of anoxygenic phototrophy and hint at the possibility of lateral gene transfer of photosynthetic operons via plasmids as observed in other marine AAP (Pradella *et al*., 2004). In addition, our results suggest that aerobic anoxygenic phototrophy may confer a pronounced physiological advantage to cells growing under the oligotrophic conditions that frequently occur in coastal marine ecosystems.

Methods

Isolation

Three HTCC strains were isolated from a depth of 10 m at a station (NH15) 27.6 km off of the coast of Oregon (44°39.1′N, 124°24.7′W) by HTC using LNHM as described previously (Cho and Giovannoni, 2004). The fresh inocula revived from frozen glycerol stocks were used throughout the study.

Polymerase chain reaction and phylogenetic analyses

Genomic DNA was extracted from 200 μl of culture using a DNeasy Tissue kit (Qiagen). A seminested fashion of

PCR was used for amplifying 16S rRNA genes and *puf*LM genes. For 16S rRNA gene sequencing, the genes were initially amplified using the primers 27F and 1492R, followed by nested PCR reaction using 27F and 1406R. To amplify *puf*LM genes, the primer pair *puf*LF (5′-CTKTTCGACTTCTGGGTSGG-3′) and *puf*MR (5′-CCATSGTCCAGCGCCAGAA-3′) was initially used for amplification (Achenbach *et al*., 2001; Beja *et al*., 2002), followed by the nested primer pair *puf*LF and *puf*MNR (5′-GTAGWACAGGTTSCCGTA-3′). Polymerase chain reaction products were purified with a PCR purification column (Qiagen) and the DNAs were sequenced by the chain termination method on an ABI 3100 automated sequencer. A touchdown PCR protocol was used to amplify the bacteriochlorophyll X gene (BchX). Using the primer pair BchlXbF (5′-GGVAARGGYGGYATYGG YAAGAG) and BchlXbR (5′-AAGCCGCCGCAVACWACR TCRCC), annealing started at 65°C and decreased by one degree for 10 rounds, then remained at 55°C for 40 additional rounds. The Platinum Hi Fidelity Taq polymerase (Invitrogen) was used. Resulting PCR products were purified by gel extraction and products were sequenced as described above. 16S rRNA gene sequences were aligned with approximately 12 000 SSU rDNA-containing database in the ARB software package (Ludwig *et al*., 1998). *puf*L sequences obtained from this study together with other sequences available in GenBank database were aligned based on their amino acid sequences with CLUSTALW embedded in BIOEDIT software (Hall, 1999) and imported into the ARB package. Only unambiguously aligned nucleotide positions were used for phylogenetic analyses. Both 16S rRNA and *puf*L phylogenetic trees were inferred with the ARB package using Juke-Cantor evolutionary distance correction and neighbour-joining analyses. The resulting 16S rRNA and *puf*L neighbour-joining trees were evaluated by bootstrap analyses based on 1000 resamplings with PAUP* 4.0 software (Swofford, 2000). *bch*X sequences were also aligned based on amino acid sequence as described above but neighbour-joining trees were constructed using the PROTDIST program embedded in BIOEDIT software and evaluated by bootstrap analysis based on 100 resamplings with the program PHYLIP. The sequences reported in this study have been deposited in GenBank under the accession numbers EF215825-EF215829.

Proteomic analyses

A representative AAP bacterium, strain HTCC2080, was grown in 20 l polycarbonate carboy under a diurnal cycle of 14 h light/10 h dark illumination from cool white fluorescent light at approximately 24 μmol m^{-2} s^{-1}. Twenty litres of early stationary-phase cells were initially harvested using a tangential flow filtration system with a 30 kDa cartridge filter in a Pellicon II Mini system (Millipore) and resulting 80 ml of final retentate solution were concentrated by centrifugation at 48.4K Xg for 1 h to produce cell pellets. Proteomic analyses were performed by LC-ESI-MS/MS as previously described (Stapels *et al*., 2004). Briefly, cell membrane material obtained from cell pellets by cell lysis, sonication and centrifugation was solubilized with 0.1% dodecyl maltoside, followed by in-solution digestion with 1 μg of trypsin at 37°C overnight. Trypsin-digested membrane material was mixed 1:1 (v/v) with solvent A (0.1% formic acid, 0.005% trifluoroacetic acid and 3% acetonitrile in H_2O) prior to injection. Chromatography was performed on a Waters CapLC system with a Symmetry 300 C18 trap (Dionex) and PicoFrit column (New Objective, 15 cm, 75 μm) packed in-house with Jupitor C5 (Phenomenex), with a 0.3 μl min^{-1} flow rate and a gradient from 3% to 90% solvent B (0.1% formic acid and 0.005% trifluoroacetic acid in 80% acetonitrile) over 70 min. The mass spectrometer was a quadrupole-time-of-flight (Q-TOF) Global Ultima system (Micromass) operated with a spray voltage of 3.5 kV. Data-dependent MS/MS was generated using a 0.5 s MS survey scan and 2.5 s MS/MS scans on the three most abundant peaks found in the survey scan. MASCOT (Perkins *et al*., 1999) (Matrix Science) software was used to search all of the tandem mass spectra.

Growth characteristics

Growth characteristics of strain HTCC2080 were examined at 16°C under different culture conditions, such as 14 h light/10 h dark (24 μmol m^{-2} s^{-1}) or permanent darkness and three different DOC concentrations (80 μM, 3.0 mM, 14.7 mM) in LNHM basal medium. Cell densities, sizes and morphology were examined at 1 day intervals by 4′,6-diamidino-2-phenylindole (DAPI) staining using a DMBR epifluorescence microscope (Leica).

Fluorescence in situ *hybridization*

Hybridizations were carried out as previously described (Morris *et al*., 2002), with the following modifications. Briefly, water from the Pacific Ocean coastal transect was collected from various depths along the Newport Hydroline (44°39.1′N) at stations NH15 (124°25′W), NH35 (124°53′W), NH65 (125°36′W) and NH127 (127°W). Hybridization reactions were performed at 37°C for 16 h in hybridization buffer containing 35% formamide and a Cy3-labelled oligonucleotide probe (NOR5–730R: 5′-TCGAGCCAGGAGGCCGCC-3′; Eilers *et al*., 2001) specific for members of the OM60 clade. Optimal hybridization stringency was achieved by washing the membranes for 10 min intervals in 70 mM NaCl, 20 mM Tris, 6 mM EDTA and 0.01% SDS. The temperature of dissociation (T_d = 50.0°C) was experimentally determined.

Cy3-positive and DAPI-positive cells based on consistent size, morphology and signal intensity criteria were counted using a Leica DMRB epifluorescence microscope, followed by calculating cell numbers of the OM60 clade by subtracting negative control counts (probe 338F) and autofluorescent cells from positive probe counts (OM60-730R).

Acknowledgements

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Neighbour-joining tree of bacterial chlorophyllide reductase subunit X (*bch*X) genes from AAP *puf* operons constructed using 100 amino acids. Bootstraps greater than 50% are shown.

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