Aerobic Anoxygenic Phototrophs of Woods Hole Coastal Waters

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Abstract

Aerobic anoxygenic phototrophs are found in marine environments worldwide and are thought to contribute significantly to the biogeochemistry and energy cycles in the oceans. These organisms are obligately aerobic bacteria that synthesize bacteriochlorophyll *a* and are related to the anaerobic purple non-sulfur photosynthetic bacteria. Aerobic anoxygenic phototrophs from the coastal waters of Woods Hole, MA, were analyzed using both culture-dependent and culture-independent methods, including PCR, spectral analysis, and fluorescence microscopy.

Introduction

Photosynthetic microorganisms in the world's oceans are responsible for roughly half the net primary productivity in the biosphere. Until recently, it was thought that most of the photosynthetic microbes in the oceans were oxygenic, and that anoxygenic photosynthesis was restricted to anoxic sun-lit environments. A growing number of organisms with hybrid photoheterotrophic metabolisms have been discovered in significant numbers in the open oceans, including bacteria with phytochrome-based and rhodopsin-based photosystems. The net contribution of these organisms indicates a more complex web of energy and carbon flow in the oceans than has previously been assumed.

Aerobic anoxygenic phototrophs (AAPs) are photoheterotrophic bacteria that were first discovered and isolated in 1979 from surfaces of aquatic plants and sand in coastal marine environments (Shiba et al.1979). In contrast to their clostest relatives, the purple non-sulfur (PNS) bacteria that synthesize bacteriochlorophyll *a* (BChl*a*) only under anaerobic conditions, the AAPs produce BChl*a* only in the presence of oxygen. Several genera, including *Erythrobacter* and *Roseobacter,* have been cultivated and have been shown to be obligate aerobes capable of using light energy for proton pumping and maintenance of an electrochemical gradient. Limited evidence also suggests some AAP isolates are capable of using light energy to fix CO_2, although photoautotrophy has not been established in any cases (Yurkov and Beatty 1998). The current view of AAP metabolism is that they use light energy to supplement cellular energy conservation (Karl 2002), though their physiology is not completely understood.

Until recently, AAPs were thought to be restricted to nutrient-rich aquatic habitats, and had not been detected in oligotrophic waters of the open ocean. In 2000, Kolber et al. reported that AAPs are widely distributed in the surface waters of the open ocean, and estimated they could constitute 11% of the total marine community and contribute up to 5% of the total surface ocean photosynthetic electron transport. A subsequent report by Beja et al. (2002) shows that AAPs in the oceans are more phylogenetically diverse than previously thought. AAPs are phylogenetically related to PNS bacteria, and all cultured representatives of the group belong to the α-proteobacteria. The report by Beja et al., however, suggests that AAPs are distributed throughout the α, β, and γ divisions of the proteobacteria.

Although more has been published recently, the role of the photosystem in these organisms is not well understood, and the conditions under which it is induced and active

have only been elucidated in a few cultivars. A better understanding of AAPs and their role in the ocean is important for a complete picture of the marine energy budget.

Methods

Sample collection. 8-liter samples were collected in acid washed plastic containers from surface waters and from a 10m depth of Vineyard Sound and Buzzards Bay, aboard the RV 'Hobbes' under Captain Alfred Spormann. Depth samples were collected using a 2L Niskin bottle.

PCR amplification of *puf* genes. PCR was used to amplify a 193bp fragment of the *pufM* gene, using primers pufM.557F (5' CGCACCTGGACTGGAC 3') and pufM.750R (5' CCCATSGTCCAGCGCCAG 3', Achenbach et al. 2001). A fragment encoding 1.5 kb of the *pufLM* operon was amplified using the same reverse primer and the pufLF forward primer (5' CTKTTCGACTGGGTSGG 3', Beja et al., 2002). Optimum annealing temperature for both reactions was 55°C.

For template DNA, 100ml of water from the surface of Buzzards Bay was filtered onto 0.2μm polycarbonate filters (Millipore GTTP). DNA was extracted from filters using the Qiagen DNeasy tissue kit, following the manufacturer's protocol for Gram-positive bacteria. A purple non-sulfur bacteria isolate was used as a positive control for *pufL* and *pufM* PCR.

Construction of *pufL* clone library. Environmental *pufL* PCR products were cloned directly into the pCR4-TOPO vector using the standard TOPO T/A cloning protocol (Invitrogen), and sequenced on an ABI 3700 automated sequencer (ABI).

Phylogenetic Methods. Environmental *pufL* gene sequences were translated into corresponding amino acid sequences using the BioEdit program, and aligned to related pufL protein sequences using the CLUSTAL-X program. Phylogenetic trees were calculated with PAUP*.

Fluorescence in situ hybridization and autofluorescence. 22.5ml of freshly collected surface water was fixed with 3.65% paraformaldehyde for 2 hours at 4°C, then filtered through 0.2μm Millipore polycarbonate filters. Filter sections were hybridized for 90 minutes at 46°C with CY3-labeled probes specific for α, β, or γ proteobacteria or a general eubacterial probe. Because the probe sequences for β and γ proteobacteria differ by only one nucleotide, unlabeled γ competitor probes were used when hybridizing with the β probe, and unlabeled β probes were used when hybridizing with the γ probe. All sections were counter-stained with 4',6-diamidino-2-phenylindole (DAPI).

Filters were viewed on the Zeiss Axioskop equipped with standard DAPI and rhodamine filters, as well as an infrared fluorescence filter cube with excitation from 350 to 550 nm, emission > 800 nm, beamsplitter 600 nm. Infrared signals were detected with a Hammatsu model C5985-02 chilled CCD camera, which has approximately 20% relative spectral response sensitivity at 850 nm. A purple non-sulfur bacteria isolate was used as a positive control for infrared autofluorescent microscopy.

Growth conditions and media. Isolates were obtained by plating seawater directly on complete sea water (CSW) media, or by filtering water through Whatman GF/F (~0.7μm) filters, then plating 100μl of filtrate on CSW plates. Plates were then incubated in the dark for 1-7 days. Pigmented colonies were re-streaked onto CSW plates, and individual colonies from each isolate were transferred to CSW broth. Individual colonies were resuspended in buffer, and 1μl of the suspension was used as template in a *pufM* PCR screen. Cultures of isolates with bands at 200bp were then scanned from 1000nm to 400nm to look for Bchl*a* peaks. Eight red-pigmented isolates had both a 200bp PCR product and *in vivo* peaks at 800 and 850nm were transferred to CSW broth and incubated with shaking in the dark until mid-log phase. Two of these were selected for further study and were inoculated into 50-ml cultures of CSW broth and dilute sea water (DSW) broth. One set of cultures was incubated with shaking in the dark, and another set was incubated with a dark:light cycle of 10h dark:14h light. 0.5ml was removed approximately every two hours for optical density measurements and *in vivo* spectral analysis. Amount of bacteriochlorophyll in cultures was normalized by dividing the peak height of the B850 peak (indicative of Bchl*a*) by the OD_{600} at that time point.

Complete sea water medium contained, per liter of filtered seawater, 5g Bacto-peptone, 3 g yeast extract, and 3ml glycerol. Dilute seawater medium contained 0.5g Bacto-peptone, 0.3g yeast extract, and 3ml glycerol per liter of seawater.

Pigment extractions; spectral analyses. Pigments were extracted from 0.5 to 1ml of culture by adding 0.5ml acetone:methanol (7:2 v/v) and sonicating at 50% output, 10x, 1sec. The lysate was centrifuged at 15000rpm for 1 minute and the spectrum of the supernatant was scanned from 1000 to 400nm. A purple non-sulfur bacteria isolate was used as a positive control for spectral analyses.

For analysis of environmental samples, 1L seawater was filtered through Whatman GF/F filters. Filters were kept at -20°C until processing. 4ml acetone:methanol (7:2 v/v) was added and samples were sonicated, centrifuged, and scanned as with cultures.

Results

Environmental samples.

Seawater samples were filtered onto 0.2μm Millipore polycarbonate GTTP filters and examined with an epifluorescence microscope with a filter set and detector capable of detecting infrared autofluorescence. Cells with autofluorescence characteristic of BChl*a* were clearly detected on the filters (figure 1). Some Chl*a*-containing cells also exhibit infrared autofluorescence in these conditions. To distinguish BChl*a* and Chl*a* autofluorescent signals, a second image was taken of the same frame with a rhodamine filter cube, which visualizes Chl*a* autofluorescence. The majority of cells exhibiting infrared autofluorescence did not exhibit autofluorescence in the rhodamine channel.

Wavelength scans of the pigment extracts from surface waters of both Vineyard Sound and Buzzards Bay showed three main peaks, at approximately 965, 665, and 435 nm (figure 2). The peak at 435nm is most likely due to carotenoids, and the peak at 665nm is characteristic of chlorophyll *a*, probably from cyanobacteria and chloroplasts.

Closer analysis of the region between 690 nm and 796 nm shows a small peak at 768nm, indicative of BChl*a*.

Of the 96 environmental *pufL* clones sequenced, 30 contained partial *pufL* genes, while the remaining clones contained only vector sequence. DNA sequences for the 30 *pufL* clones were 99% identical over 750 base pairs with quality scores >20. Corresponding deduced amino acid sequences were also nearly identical. A representative *pufL* deduced amino acid sequence, clone WH-B06, was aligned to related sequences downloaded from GenBank (figure 3). Phylogenetic analysis shows that WH-B06 is most closely related to *pufL* genes from organisms in the *Roseobacter* genus, a subset of the α-proteobacteria division in the bacterial domain (figure 4).

Cultures.

Seventy-seven pigmented isolates were screened by PCR and wavelength scans. The colors in this group included beige, yellow, pink, and orange. Only a few of these isolates had any PCR product in the *pufM* amplification reaction, and of these, several of the products were significantly larger than 200bp. None of the *in vivo* wavelength scans of these isolates showed significant peaks at 800 or 850nm; however, in some species, pigments are only produced under particular conditions which may not have been met in our culture regime.

Roughly ten red-pigmented isolates were obtained per 100ml of filtered seawater. Eight of these were inoculated into CSW broth and grown to mid-log phase, then two were selected for further study. AAP 78 and AAP 80 were both small, non-motile rods (figure 5). These were inoculated into complete sea water medium (CSW) and dilute sea water medium (DSW) and grown either in the dark or in a 14h light: 10h dark cycle. Cultures grown in CSW grew faster than those in DSW, and after 49 hours if incubation were nearly an order of magnitude more dense than those grown in DSW (figure 6A, 6B). Although the cultures in CSW were a deeper red color than those in DSW (figure 7), the populations in the dark produced more bacteriochlorophyll per cell during the first 24 hours of the growth curve. After approximately 24 hours of incubation, all populations produced approximately the same amount of bacteriochlorophyll per cell (figure 6C, 6D).

Fluorescent in situ hybridization with these two cultures suggests that AAP 78 is a member of the γ-proteobacteria and AAP 80 belongs to the α-proteobacteria. These isolates fluoresce in the infrared range, confirming that they have BChl*a* (figures 8 and 9).

Pigment extractions from AAP 78 and AAP 80 confirm the presence of BChl*a*. Both cultures have BChl*a*-specific peaks at 770nm and 480nm, as well as a peak at 963nm (figure 10).

Discussion.

The presence of bacteriochlorophyll *a* and *pufL* and *pufM* genes closely related to those of *Roseobacter* species in the surface waters off of Cape Cod indicates that aerobic anoxygenic phototrophs (AAPs) inhabit this environment. Purple sulfur bacteria and purple nonsulfur bacteria also produce BChl*a*, but these groups do not live in the oxic zone. To date, the only aerobes that have been shown to produce BChl*a* are the AAPs. In addition, the 30 *pufL* genes cloned from the surface waters near Woods Hole, MA, taken

8 days apart, were nearly identical, suggesting that one phylotype dominates the surface AAP population during this time.

All representative AAPs that have been cultured to date belong to the α-subclass of proteobacteria; however, sequences have been obtained from environmental BAC clones containing *puf* operons and rRNA operons that indicate that some AAPs may belong to the β and γ subclasses. Results from fluorescent in situ hybridization suggest that AAP 78 is an α-proteobacterium and AAP 80 could be a γ-proteobacterium. These results will soom be confirmed by 16S rDNA sequencing. If AAP 80 is indeed a γ–proteobacterium, it is one of the first cultured representatives of AAPs in this group.

Although it might be expected that organisms producing bacteriochlorophyll and LH1 would grow better in the light, our results indicate that AAPs grow as well in the dark as in the light and may produce more pigment per cell in the dark. Previous work has shown that AAPs produce bacteriochlorophyll in the dark, but its production is inhibited in low-light environments. In addition, they suggest that pigment production may be a response to low carbon rather than high light conditions (Kolber et al. 2001, Suyama et al. 2002, Yurkov and Beatty 1998). Our results suggest that isolates AAP 78 and 80 may have a similar pattern: during the first 24 hours of the growth curve, more bacteriochlorophyll was produced per cell in the DSW cultures than in the CSW cultures, which had ten times the amount of carbon. These experiments will also be repeated, using OD_{660} as a measure of cell density and using more stringent culture conditions.

Analysis of the pigments in both cultures showed characteristic Bchl*a* peaks at 803 and 850nm *in vivo* and at 770nm in acetone:methanol extracts. Additionally, AAP 78 showed a pronounced peak at 963nm after 4.5 hours of growth. After 45 hours of growth, this peak had disappeared. Since a peak appears at this position in spectra of both *in vivo* cultures and methanol extracts, it is probably not related to pigments; however, it should be noted that a purple sulfur bacterium has been isolated that produces Bchl*a* and has an *in vivo* peak at 963nm. The authors suggest that this peak may be due to a novel arrangement of the antenna pigments and proteins (Permentier et al. 2001). They also note that 963nm is very close to the absorbance maximum of water at 975nm, suggesting that this light-harvesting system would not be advantageous far below the surface of the water.

Aerobic anoxygenic phototrophs have been shown to be important members of the surface marine phytoplankton community in the open ocean. Several lines of evidence, including pigment analysis, a clone library, and direct isolation, now indicate that a significant population of AAPs also inhabits near-shore waters. This population is most likely dominated by one phylotype; however, it is composed of more than one species, including α-proteobacteria similar to *Roseobacter* and also γ-proteobacteria, which have been detected but not cultivated before. In culture, these organisms produce BChl*a* under aerobic conditions in the dark, especially when carbon is a limiting factor. Since pigment production in AAPs seems to be more related to carbon concentration than available energy from light, the role of the photosynthetic apparatus in these organisms is a conundrum that should be investigated further.

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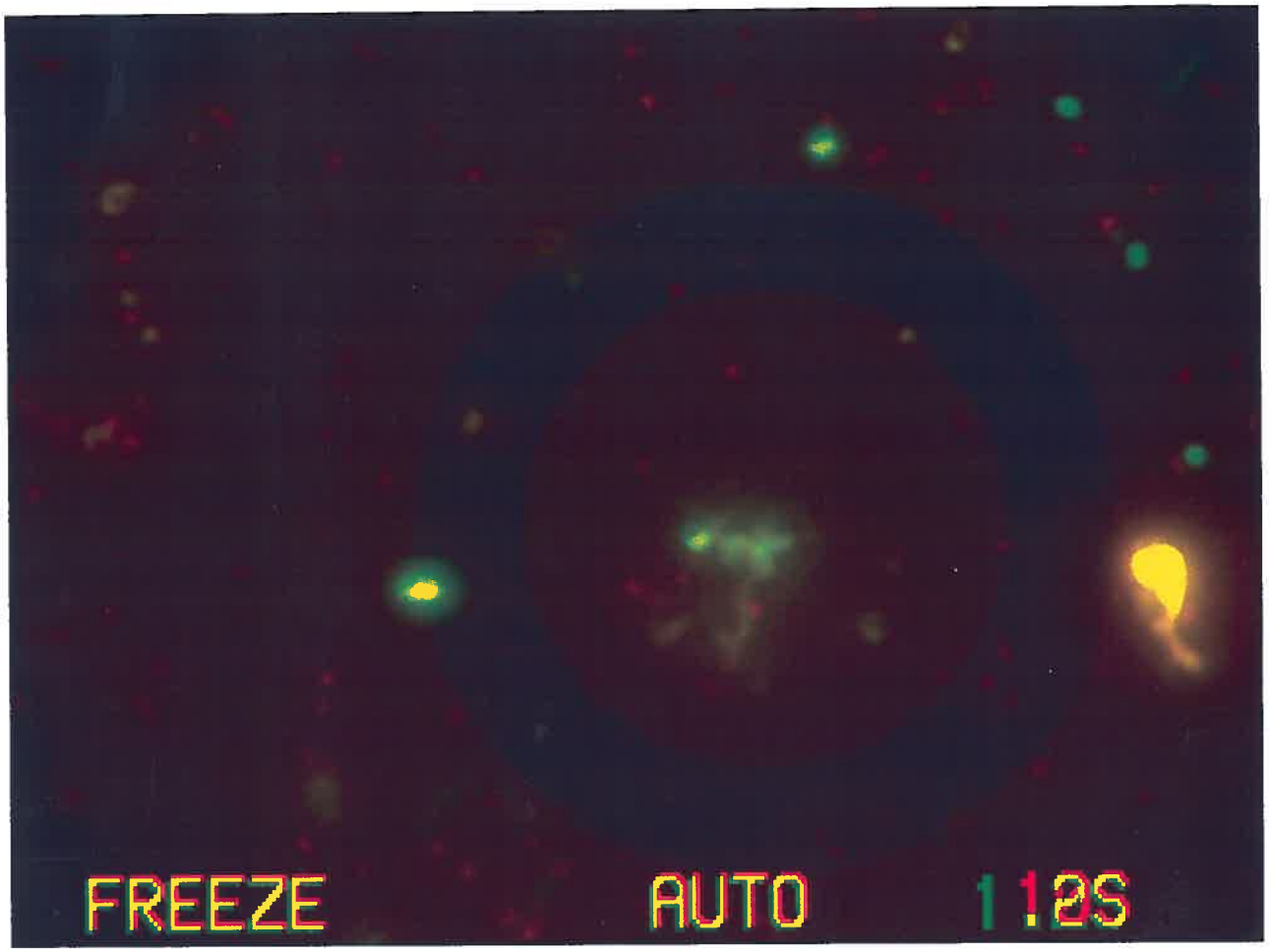


Figure 1. Overlay micrograph of BChla infrared autofluorescence (red) and Chla autofluorescence (green) from environmental sample (22.5ml seawater filtered onto 0.2µm filter). Some cells (yellow) may contain both pigments.

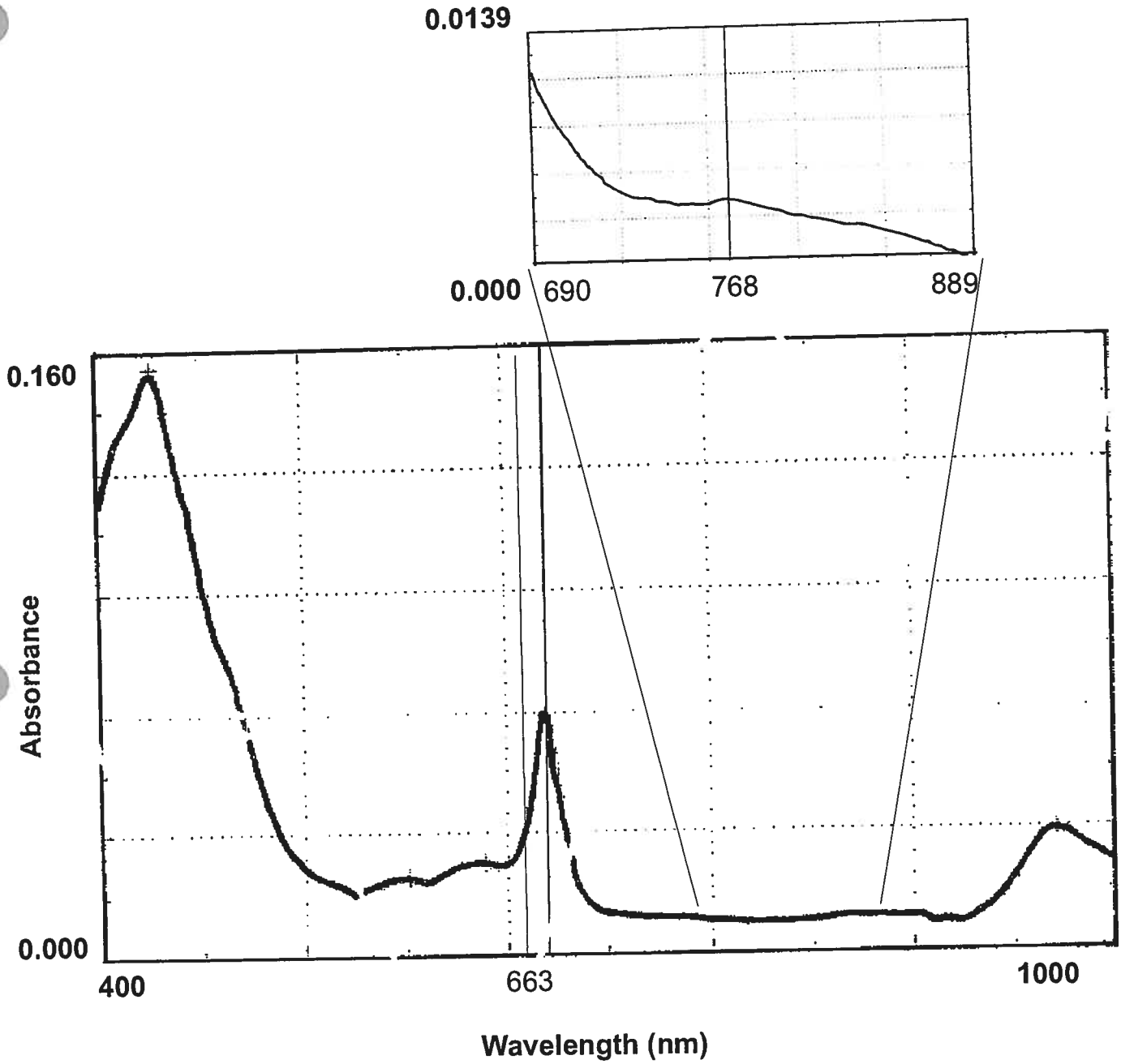


Figure 2. Wavelength scan of methanol:acetone extract from 100ml surface water filtered onto 0.2µm filters. Large peak at 665nm indicates Chla; small peak at 768 nm is characteristic of BChla.

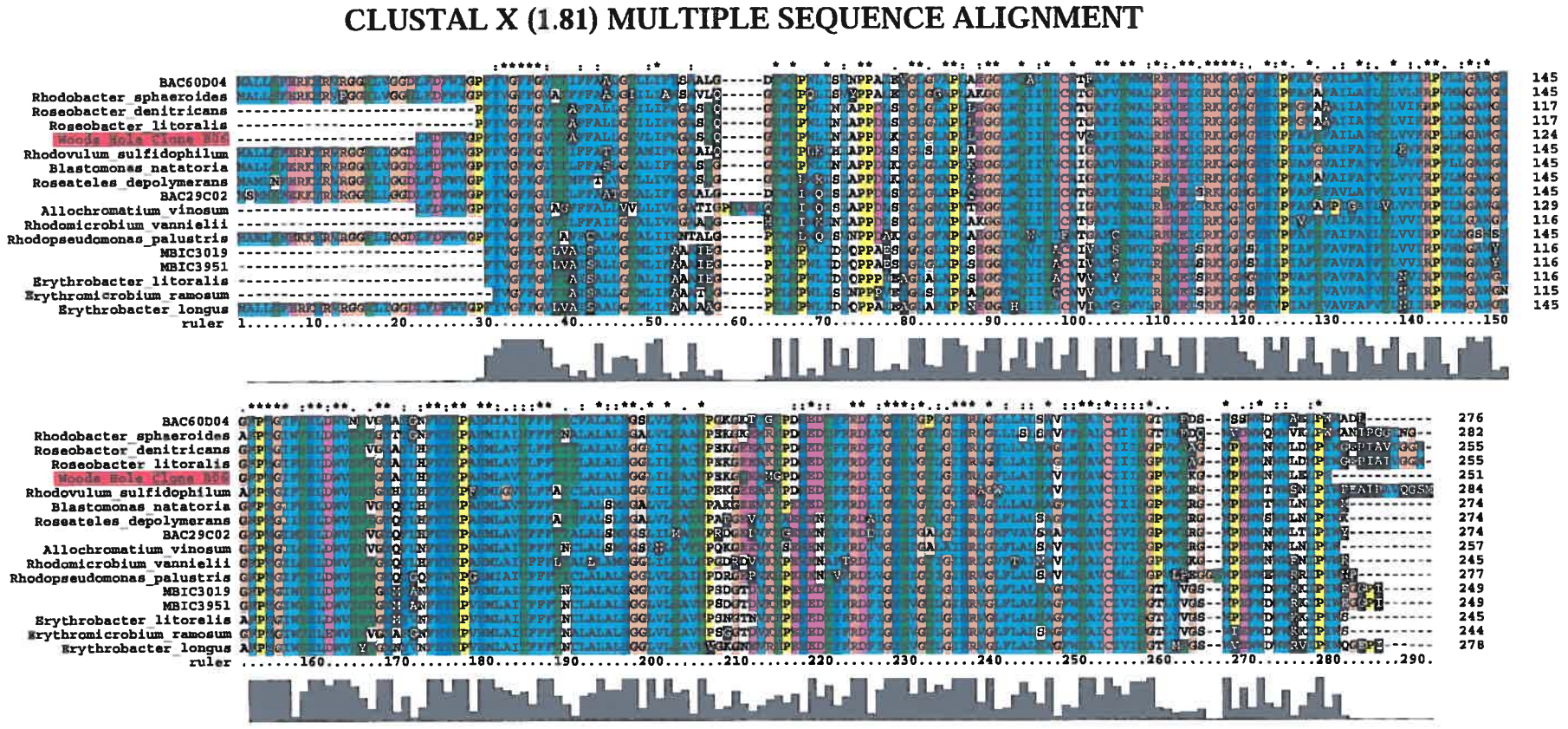


Figure 3. Alignment of pufL amino acid sequence of Woods Hole Clone B06 (red) and related proteobacteria.The pufL sequence from the green non-sulfur bacterium *Chloroflexus aurantiacus* was used as an outgroup in subsequent phylogenetic analysis.

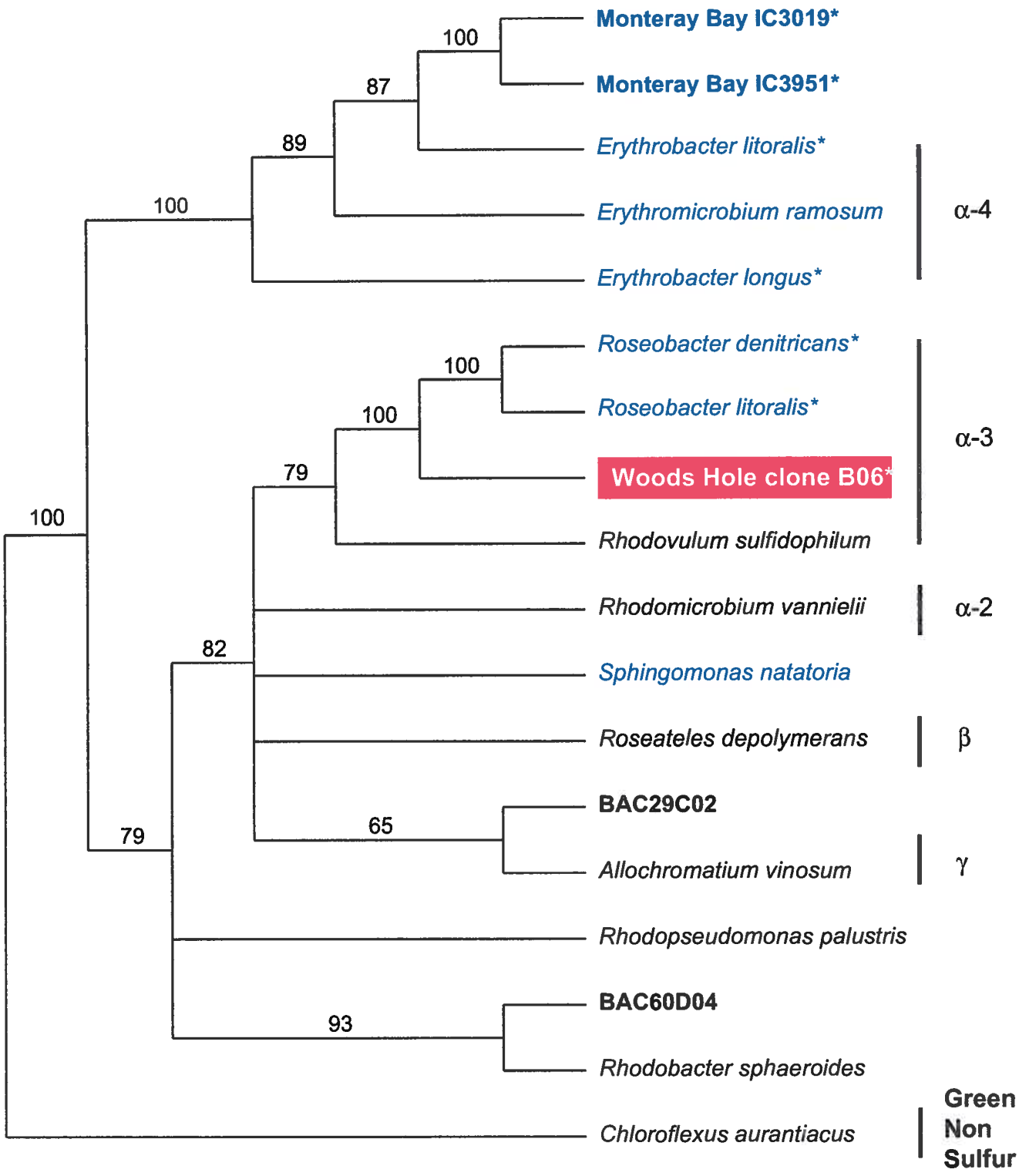


Figure 4. Bootstrapped neighbor-joining tree of pufL proteins, including Woods Hole Clone B06 (red). Species in blue are cultivated; asterisks indicate a marine isolate. Bold-face indicates sequences from environmental BAC clones (Beja et al. 2002).

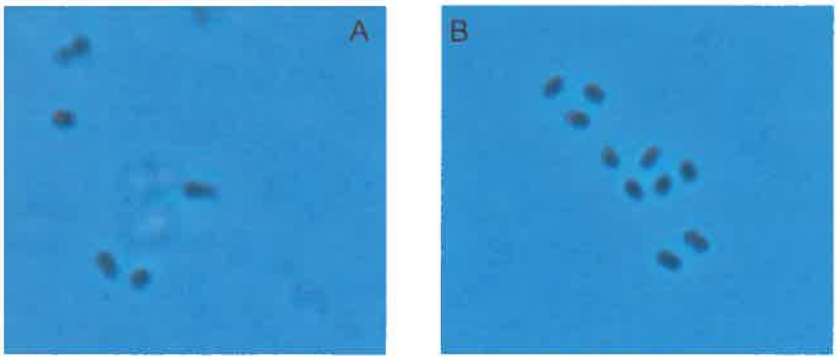


Figure 5. Light micrographs of isolates AAP 78 (A) and AAP 80 (B).

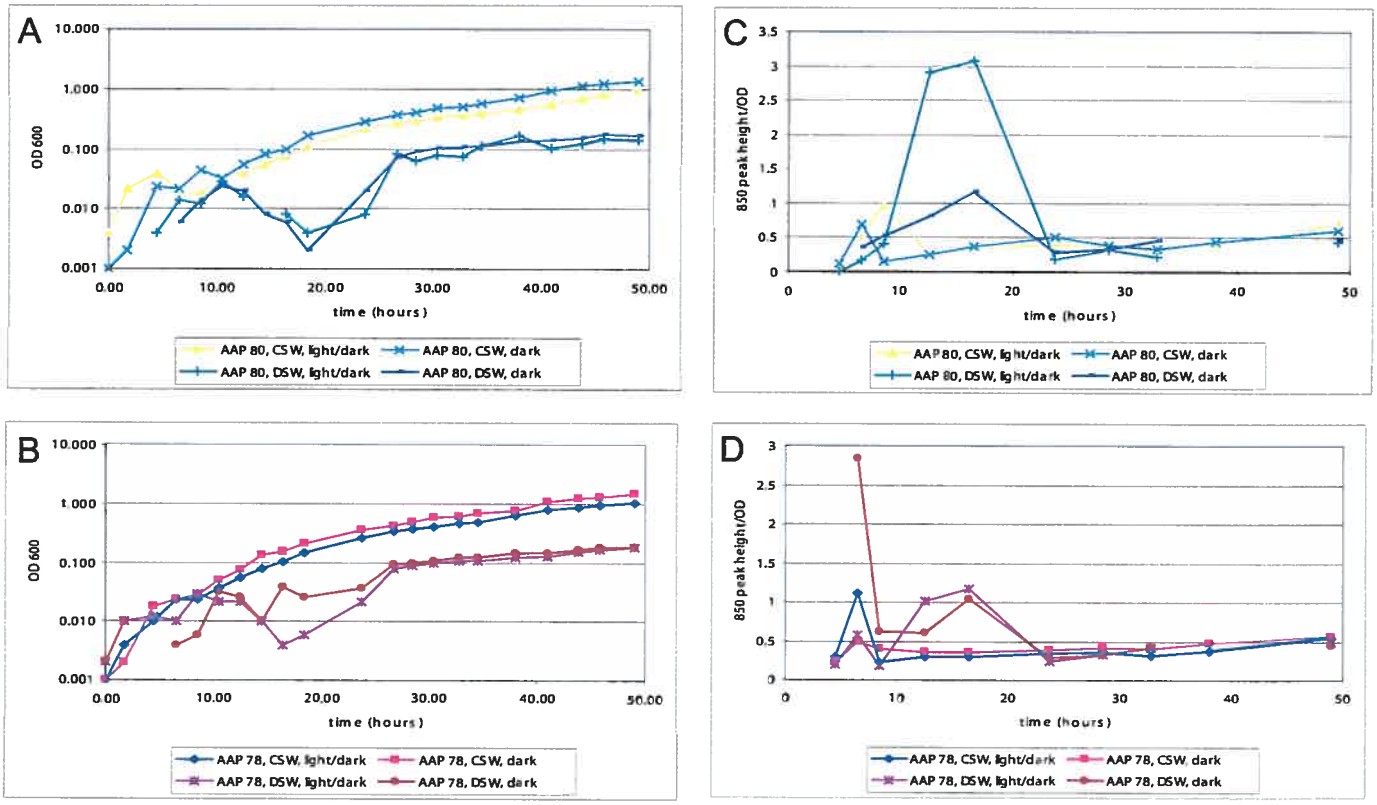


Figure 6. A and B: Growth curves showing optical density of isolates AAP 78 and AAP 80 in complete and dilute seawater media. C and D: bacteriochlorophyll a production normalized by OD in each culture.

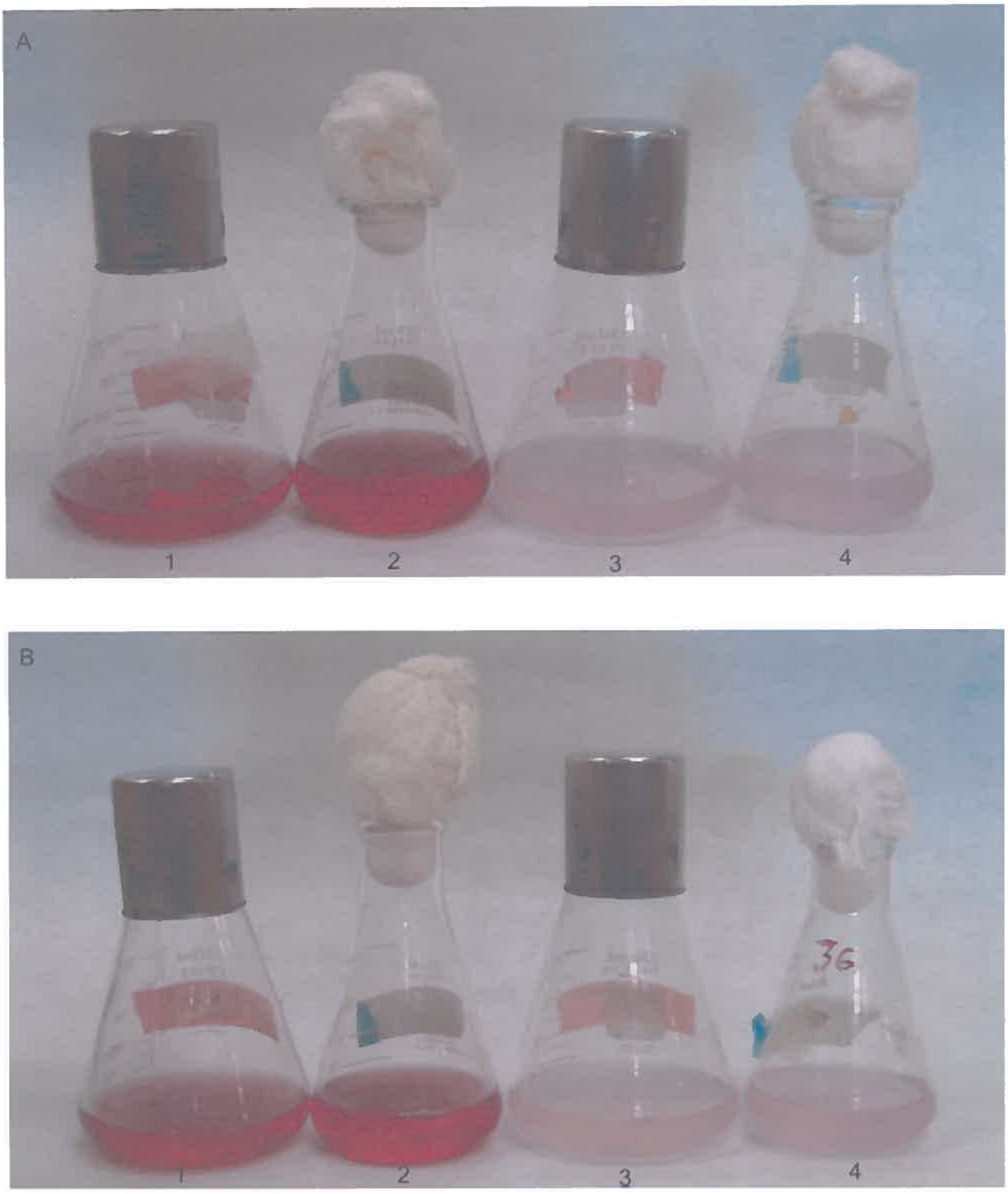


Figure 7. Ciltures of AAP 78 (A) and AAP 80 (B). Flasks 1 and 3 were incubated with a 14hour light-10 hour dark cycle; flasks 2 and 4 were incubated constantly in the dark. Flasks 1 and 2 contain complete seawater medium, and flasks 3 and 4 contain dilute (0.1X) seawater medium.

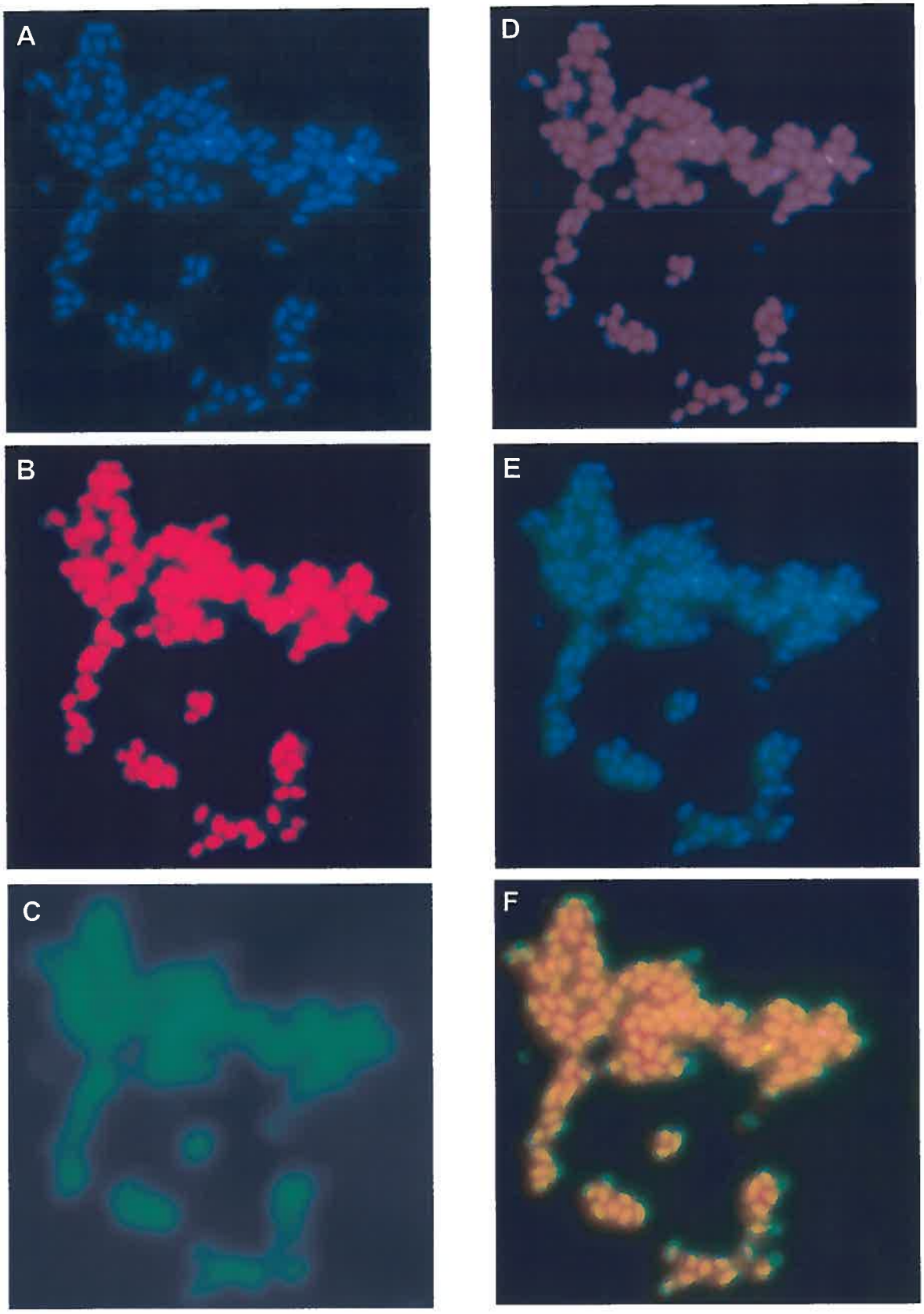


Figure 8. Epifluorescent micrographs of isolate AAP 78. (A) DAPI stain. (B) Cy3 labeled gamma proteobacteria FISH probe. (C) Infrared autofluorescence. (D) DAPI and Cy3 FISH overlay. (E) DAPI and infrared autofluorescence overlay. (F) DAPI, Cy3 FISH and infrared autofluorescent overaly.

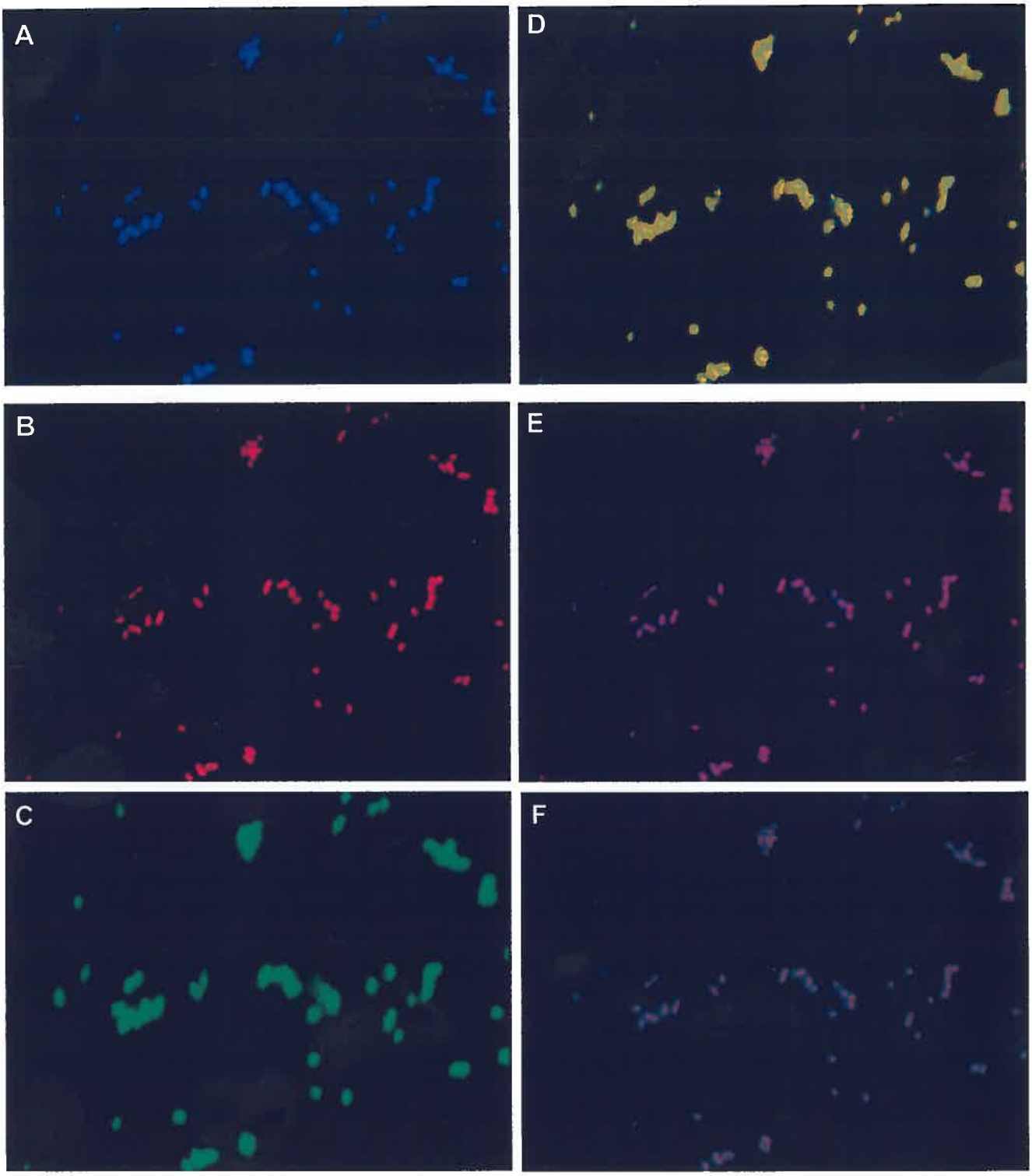
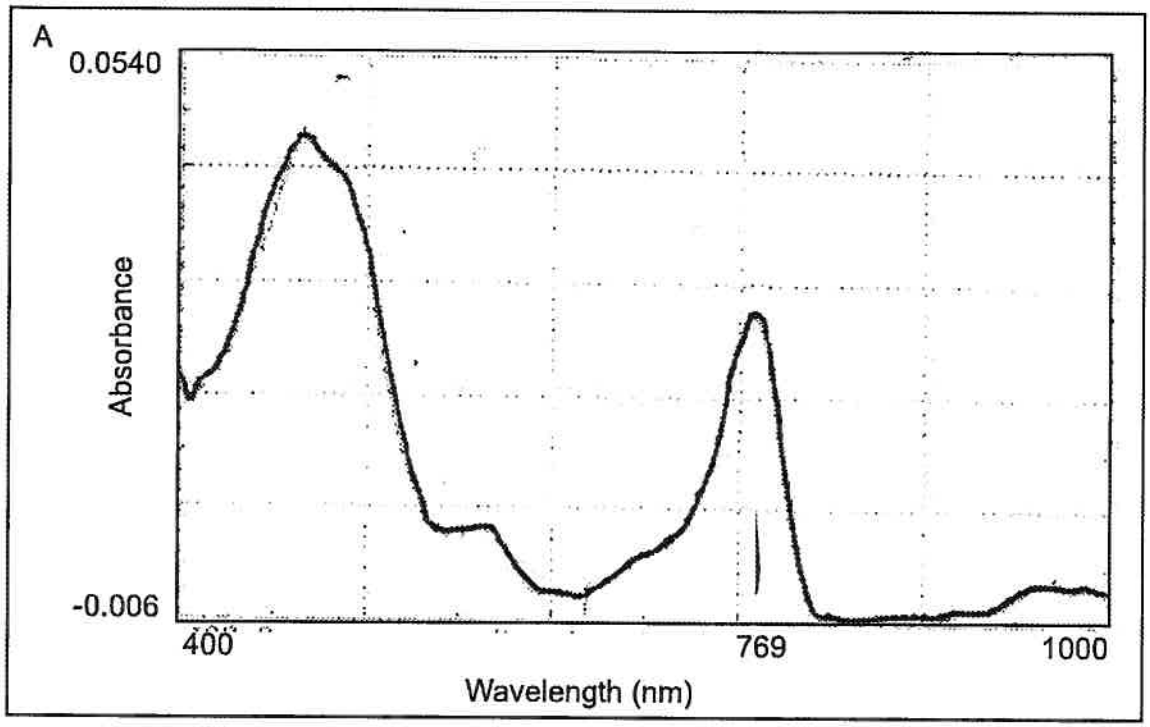


Figure 9. Epifluorescent micrographs of isolate AAP 80. (A) DAPI stain. (B) Cy3 labeled gamma proteobacteria FISH probe. (C) Infrared autofluorescence. (D) DAPI and Cy3 FISH overlay. (E) DAPI and infrared autofluorescence overlay. (F) DAPI, Cy3 FISH and infrared autofluorescent overaly.



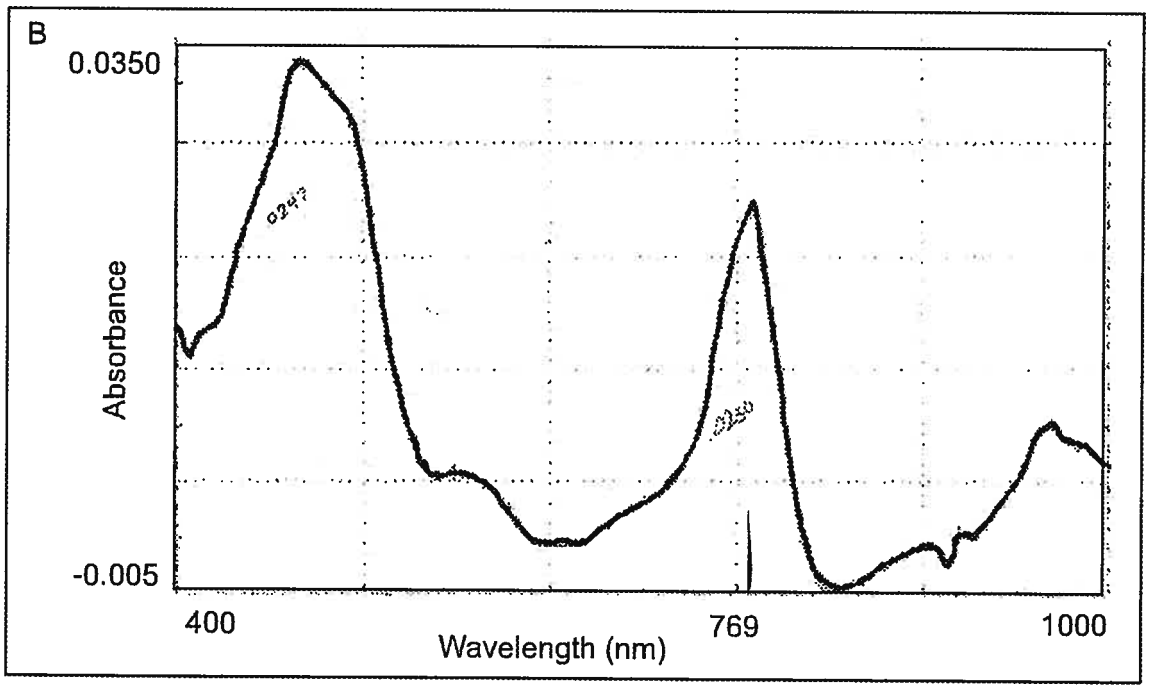


Figure10. Absorbance spectra in methanol:acetone of AAP 78 (A) and AAP 80 (B). Peak at 770nm is characteristic of BChla.