Diversity of the Aerobic Phototrophic and Heavy Metalloid Reducing Bacteria: Perspectives Gained from the Study of Novel Isolates.

By

Christopher Rathgeber

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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Abstract.

The research presented focused on the isolation of bacteria from extreme environments and their characterization in terms of phenotype, phylogeny, photosynthetic function and ability to reduce heavy metalloids. Isolates from the meromictic Mahoney Lake and deep ocean hydrothermal vents were examined using classical techniques and several were described taxonomically. The Mahoney Lake strains represented diverse new members of the aerobic phototrophic and the purple non-sulfur bacteria, produced unusual pigment protein complexes and were adapted to the fluctuating levels of pH and salinity in a meromictic lake. The metalloid resistant isolates also tolerated fluctuating conditions, and reduced TeO_3^{2-} or SeO_3^{2-} accumulating elemental Te or Se.

Roseicyclus mahoneyensis gen. nov., sp. nov was proposed for ten vibrioid strains of aerobic phototrophic bacteria with an unusual monomodal light harvesting II complex (805-806 nm), and tolerance to high salinity. Four appendaged and budding bacteria were closely related to the genera *Erythrobacter* and *Porphyrobacter*. Although 16S rRNA phylogeny could not resolve the relationship between these two genera, the new isolates shared several phenotypic traits with the genus *Porphyrobacter* and thus were proposed as a new species, *P. meromictius*. Phylogenetic analysis of metalloid-reducing strains identified them as relatives of *Pseudoalteromonas*. However they had important differences from previously described species and thus were proposed as *P. telluritireducens* sp. nov. and *P. spiralis* sp. nov.

In order to determine the distribution of aerobic anoxygenic phototrophs in the ocean around black smokers the cultivable population was investigated, revealing that *C. bathyomarinum* can be found at depths of 500 m and below, but not at the surface. The

pigment protein complexes of this species were capable of light induced e^- transfer and thus *C. bathyomarinum* is photosynthetically competent and should be able to supplement its energy needs when light is available.

Photosynthetic e^- transfer was investigated in *P. meromictius*, ML31 and *R. mahoneyensis*, $ML6^T$. Photochemical activity in ML31 was observed aerobically, and the photosynthetic apparatus was not functional under anaerobic conditions. In $ML6^T$ low levels of photochemistry were measured anaerobically, however, electron transfer occurred optimally under low oxygen conditions, an interesting and unique case.

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List of abbreviations.

AnAP	anaerobic anoxygenic phototrophs
APB	aerobic anoxygenic phototrophic bacteria
BChl	bacteriochlorophyll
BSA	bovine serum albumin
CEA	Commissariat à l'Énergie Atomique
Chl	chlorophyll
CTD	conductivity, temperature, depth
cyt	cytochrome
DSRV	deep submergence research vehicle
e^-	electron
EMBL	European Molecular Biology Laboratory
EPS	exopolysaccharide
HPLC	high performance liquid chromatography
ICM	intracytoplasmic membrane
IREM	infra-red epifluorescence microscopy
IRFRR	infra-red fast repetition rate
LDAO	lauryldimethylamine oxide
NASA	National Aeronautics and Space Administration
NSERC	Natural Science and Engineering Research Council
NSF	National Science Foundation
P	primary electron donor
PCR	polymerase chain reaction

PMSF	phenylmethylsulfonyl fluoride
PSM	photosynthetic membranes
LH	light harvesting complex
Q_A	primary electron acceptor
RC	reaction center
RDP	Ribosomal Database Project
R/V	research vessel
TEM	transmission electron microscope
TMBZ	3,3',5,5'-tetramethylbenzidine

Chapter 1.

Introduction.

1.1. Microbial diversity of extreme environments.

1.1.1. Towards a definition of "extreme".

Extreme environments are most often defined as ecological niches having at least one physical or biogeochemical parameter that is "abnormal", or has a limiting effect on life. In microbiology, this usually refers to the environmental conditions that differ greatly from the growth conditions of most laboratory strains. The term extremophile is frequently used for organisms that thrive in such habitats (Macelroy, 1974), and the term extremotroph can be applied to describe those that simply tolerate one or more extreme parameters, although they might prefer moderate conditions. The definition of an abnormal parameter is not well defined, but has been suggested to be any condition that is not "moderate" (Das et al., 2006) or "beyond a normal acceptable range" (Satyanarayana et al., 2005).

A better definition for an extreme parameter is a condition that inhibits growth of the non-extremophile by acting non-specifically on a broad range of cellular targets, as opposed to a toxin that affects one or few specific sites (Nies, 2000). With this in mind, the most commonly recognized extreme factors (and corresponding extremophiles) are high temperature (thermophiles and hyperthermophiles), low temperature (psychrophiles), high pressure (barophiles or piezophiles), dessication (xerophiles), water activity (osmophiles), salinity (halophiles), low pH (acidophiles), high pH (alkaliphiles) and radiation (radiophiles) (Rothschild and Mancinelli, 2001). Additionally, chemical extremes are sometimes considered. For example, high concentrations of heavy metals are known to inhibit the growth of cells with non-specific action (Nies, 1999), and thus

organisms that thrive under heavy metal rich conditions could be considered "metalophiles" (Nies, 2000). Using this approach it is possible to include other less exceptional parameters, such as nutritional conditions and oxygen tension (Rothschild and Mancinelli, 2001) in a definition of an extreme environment.

1.1.2. The meromictic Mahoney Lake.

In meromictic lakes some portion remains unmixed with the main water mass (Northcote and Halsey, 1969), and typically such lakes can be divided into the monimolimnion (the anaerobic bottom waters that do not undergo seasonal mixing with the upper part due to high chemical density) and the mixolimnion (the circulating and oxygenated portion). The monimolimnion and mixolimnion are separated by the chemocline, a line that represents the area of steepest chemical gradient. The state of meromixis is usually provoked by the chemical density gradients that prevent the monimolimnion and mixolimnion from mixing.

The meromictic Mahoney Lake is located in the dry south central region of British Columbia (Fig. 1.1). Lack of outflow, shelter from prevailing winds and a watershed of soft, fractured and alkali lava rock, has resulted in high concentrations of the cations Na^+, Ca^{2+} and Mg^{2+} and the anion SO_4^{2-} (Northcote and Hall, 1983) in the monimolimnion. Meromixis is maintained by a sharp chemical discontinuity between the monimolimnion, which may have salinity as high as 40‰, and the mixolimnion, which has salinity of about 4‰ (Hall and Northcote, 1986). Mahoney Lake can be considered

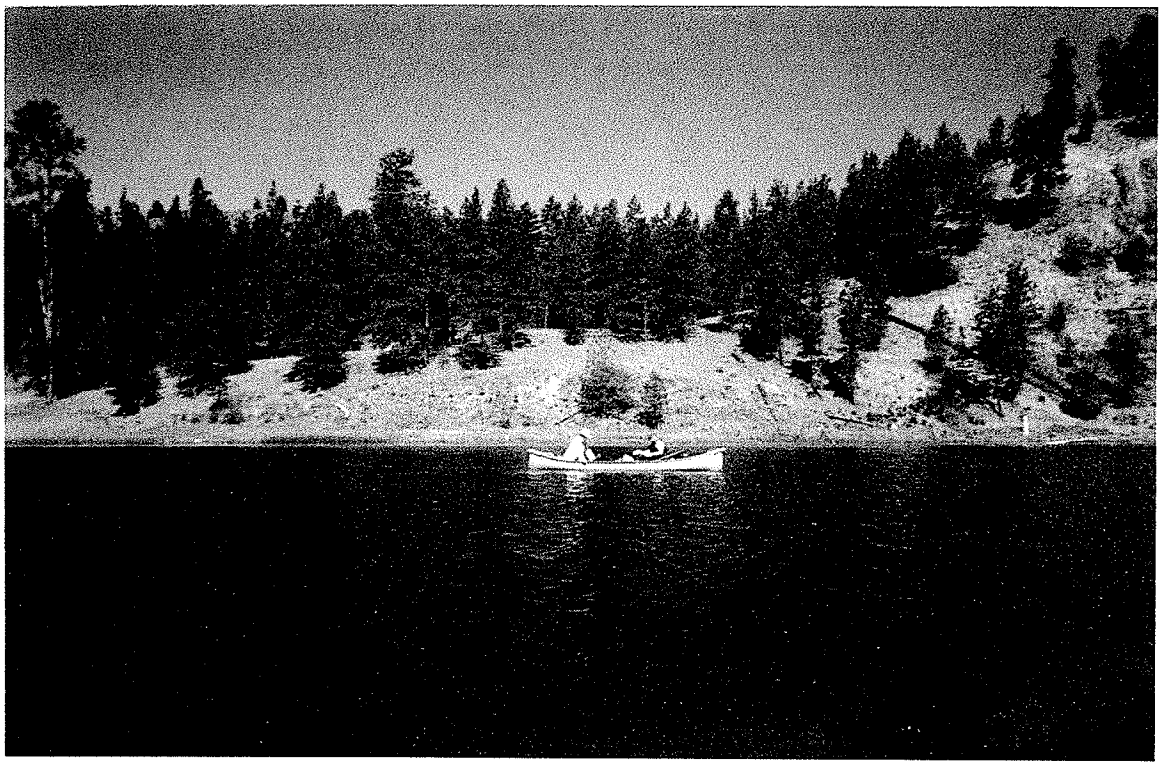

Fig. 1.1. Photograph of Mahoney Lake, a meromictic lake in south central British Columbia.

an extreme environment based primarily on two parameters. High ionic concentrations, including unusually elevated levels of the anion SO_4^{2-} (Hall and Northcote, 1986) should favor halotolerancy or halophily of the inhabitants, and relatively high alkalinity, with pH ranging between 7-9 should favor alkaliphily. Because of these harsh conditions, Mahoney Lake is marked by the absence of higher plants and animals. However, a rich ecosystem exists, dependent on the activity of microbial photosynthetic primary producers, including purple sulfur and non-sulfur bacteria, green sulfur bacteria, some cyanobacteria and few eukaryotic algae (Northcote and Halsey, 1969; Overman et al., 1991).

1.1.3. Deep ocean hydrothermal vents of the Juan de Fuca Ridge.

Deep ocean hydrothermal vent systems were first discovered in 1977 (Weiss et al., 1977) along the Galapagos Rift, and over the next two decades were identified at several other sites along tectonic plate boundaries (Van Dover, 2000). Such areas are characterized by a high degree of geothermal activity resulting in output of high temperature, acidic and mineral rich effluents. These so-called "black smokers" frequently contain high levels of sulfides that precipitate upon contact with much cooler surrounding sea waters, which form steep-walled sulfide structures up to 20 m in height (Delaney et al., 1992), and produce black particulates in the rising hot waters (Fig. 1.2).

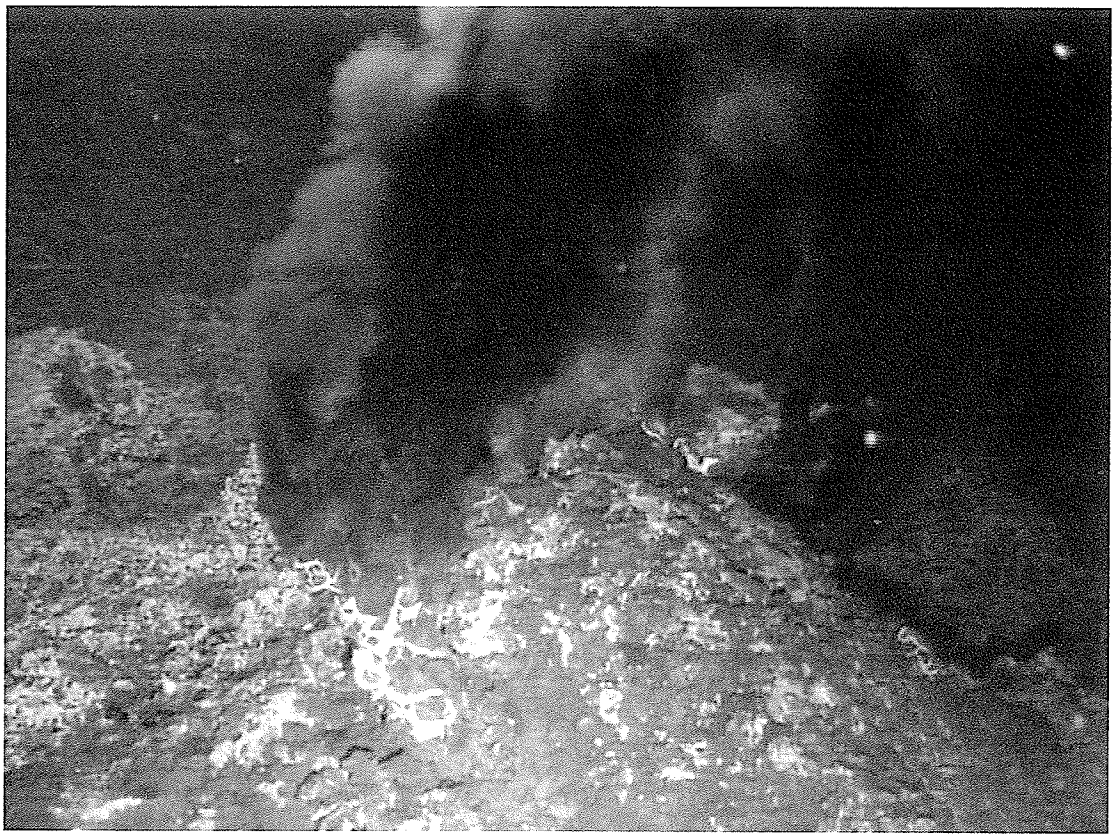

Fig. 1.2. Photograph of a black smoker situated in the Main Endeavour Segment of the Juan de Fuca Ridge in the Pacific Ocean.

Around the hydrothermal vents exists an abundance of biological diversity, in stark contrast to the deep ocean lacking geothermal activity. The life consists of animals (tube worms, clams, crustaceans, gastropods and limpets) as well as a plethora of microorganisms and likely represents the most productive ecosystem independent of photosynthetically fixed organic carbon (Van Dover, 2000). Indeed hydrothermal vent communities were once thought to exist in total darkness, however, light in the form of geothermal black-body radiation has been detected emanating from black smoker chimneys (Nisbet et al., 1995; Van Dover et al., 1996). This discovery led to the suggestion that bacterial photosynthesis may occur in the vicinity of the vents (Van Dover et al., 1996).

Hydrothermal vents present several conditional challenges to microbial life. Effluent waters can reach 350 °C (Delaney et al., 1992), and quickly cool in contact with the cold deep ocean, creating a steep gradient. The hottest areas make an ecological niche for thermophiles and hyperthermophiles, and cooler areas are likely an important niche for thermotolerant organisms. Enriched levels of toxic sulfide and heavy metals (Butterfield et al., 1994) ejected from vents suggest that the organisms must also develop chemical tolerance, therefore such communities are ideal locations to search for metal-resistant species. Additionally, the absence of sunlight, and presence of only minute levels of geothermal radiation (Van Dover et al., 1996), not extreme by the definition of Nies (2000; discussed above), can be seen as such in the context of a very productive ecosystem. For this reason hydrotherms grant an opportunity to search for extreme phototrophic organisms.

1.1.4. Microbial diversity in extreme environments

The importance of investigating microbial diversity of extreme environments cannot be understated. Extremophilic and extremotolerant microorganisms are valuable resources for the exploitation of biotechnological processes, and excellent models to study how biomolecules are stabilized under extremes of condition (Aguilar, 1996; Guzennec, 2002). Because organisms have had to adapt to one or several physical or chemical extremes, they allow us to study how such parameters affect cells, and examine evolution in response to environmental stresses (Tehei and Zaccai, 2005). Extremophilic enzymes are of great value because of the potential for high stability under a variety of industrial processes, and utilization of extremophilic species reduces risk of contamination (Aguilar, 1996). Some extremophiles and their enzymes have already had significant economic impact in science and biotechnology. There is no better example than the DNA polymerase of the hydrothermal vent thermophile *Thermus aquaticus*, which functions and maintains stability at high temperatures used in PCR and is, therefore, essential to modern molecular biology (Aguilar, 1996). However, despite the potential value of organisms, microbial diversity of extreme habitats is relatively unexplored (Tehei and Zacai, 2005), and as such offers virtually unlimited opportunity for new discoveries.

1.2. Significance of discovering new strains.

Although the modern study of microbiology is approaching its third century and vast collections of microorganisms have been assembled (DSMZ or ATCC, for example), exploration of the richness of microbial life is still in its infancy. Several thousand

species of prokaryotes have been described taxonomically, however, estimates of the total number of species that exist on the Earth based on molecular techniques suggest greater than 1 million (Schleifer, 2004). This realization naturally leads to the questions: "Who are they?" and "What are they doing?"

The development of molecular methods in microbial ecology has had a profound effect on our appreciation of diversity. Research relying on phylogenetic analysis of 16S rRNA genes extracted from natural samples brings a tendency to entirely replace attempts to culture novel organisms. In fact, the growing emphasis placed on molecular methods over classical microbiological techniques has led some scientists to question the necessity of working with pure cultures at all (Leadbetter, 2003). Although molecular approaches have been a great asset to modern microbiology, allowing preliminary determination of physiology and recognition of genetic diversity in communities independent of culturing, they are not sufficient to answer the questions raised above.

The isolation of pure cultures is still paramount to the study of microbiology. Only through laboratory-based investigation of strains can we fully appreciate how enzymes function in unison to give an organism a particular set of physiological traits and to influence how it will behave in nature. Isolation of new species is still the primary method to discover novel enzymes and biological processes of ecological significance, evolutionary importance and industrial value. Furthermore, the process of cultivating and isolating followed by taxonomic description of new strains, satisfies the basic human tendency to organize our knowledge (in this case knowledge of microorganisms) using a coherent and useful system, and makes it available to the scientific community.

1.3. The aerobic phototrophic bacteria.

For many years it was generally believed that anoxygenic photosynthesis was an anaerobic growth mode of either obligately, or facultatively anaerobic bacteria capable of switching between respiration under aerobic conditions and phototrophy under anaerobic conditions (Pfennig, 1978). In most species of facultatively anaerobic phototrophic bacteria, the synthesis of the photosynthetic apparatus is induced by oxygen deprivation and modulated by light intensity (Bauer et al., 1993; Oh and Kaplan, 2001).

In 1979, the first reported member of the aerobic anoxygenic phototrophic bacteria (APB), *Erythrobacter longus,* (Shiba et al., 1979; Shiba and Simidu, 1982), discovered in the Bay of Japan, was shown to contradict conventional knowledge of the phototrophic bacteria, producing its photosynthetic apparatus only in the presence of oxygen and the absence of light (Shiba, 1984). Since that time many other APB have been described from a wide variety of both saline and freshwater habitats, soils, acid mine drainage sites and soda lakes (Yurkov and Beatty, 1998a; Yurkov, 2001; Rathgeber et al., 2004; Yurkov and Csotonyi, 2006). The distinguishing features of this increasingly large group of bacteria are: the presence of bacteriochlorophyll (BChl) *a* incorporated into light harvesting (LH) and reaction center (RC) complexes capable of transforming light into electrochemical energy under aerobic conditions; a high midpoint potential of the RC primary electron acceptor (Q_A); a relatively low amount of photosynthetic units per cell; extreme inhibition of BChl synthesis by light; an abundance of carotenoid pigments; and the inability to grow phototrophically under anaerobic conditions (Yurkov and Beatty, 1998a).

The APB are made up of phylogenetically diverse members interspersed predominantly throughout the α-subclass of the *Proteobacteria*, closely related to anoxygenic phototrophic purple non-sulfur bacteria as well as chemotrophic species (Yurkov and Beatty, 1998a). The recently described *Roseateles depolymerans* belongs to the β-subclass of the *Proteobacteria* (Suyama et al., 1999), thus increasing the phylogenetic diversity of this group.

1.3.1. Ecological perspectives.

The first report of obligately aerobic, BChl *a*-containing bacteria came almost 30 years ago. Since that time, the APB have been found to arise from a variety of natural and anthropogenic habitats. The search for APB has mainly focused on five ecosystems: marine surface and deep ocean waters; inland saline and soda lakes; hot springs; freshwater ponds and rivers; and soils (Rathgeber et al., 2004) (Table 1.1).

The majority of work has been done on saline environments, mostly eutrophic in nature. Several marine locations have been studied, including both surface and deep ocean waters. The first APB discovered, *E. longus*, *Roseobacter litoralis* and *Roseobacter denitrificans* were found in seawater, sand and bottom sediments of Tokyo Bay, Japan (Shiba et al., 1979; Shiba, 1991). Several BChl *a*-containing strains isolated from marine environments along the east and west coasts of Australia (Shiba et al., 1991) were later divided into four phenotypic and several genotypic groups (Nishimura et al., 1994). Ten of these strains were classified as two species within a new genus, *Roseibium denhamense* and *Roseibium hamelinense* (Suzuki et al., 2000). Another marine species, *Erythrobacter litoralis* was discovered shortly thereafter from a cyanobacterial mat in the

Table 1.1. Some determinative characteristics of genera containing APB.

Genus (described by)	Habitat and site of isolation	Color	Cell shape	*In vivo* Bchl peaks (nm)
Acidiphilium (Wakao et al., 1993)	Acid mine drainage	Red-pink	Rod	792, 864
Acidisphaera (Hiraishi et al., 2000)	Acidic hot springs and acid mine drainage	Pink to salmon-pink	Coccoid to short ovoid rods	801, 874[1]
Citromicrobium (Yurkov et al., 1999)	Deep ocean hydrothermal vent plume waters	Citron yellow	Pleomorphic	800, 867
Craurococcus (Saitoh et al., 1998)	Soil	Pink	Coccoid	800, 872
Dinoroseobacter (Biebl et al., 2005)	Marine	Beige to light red	Coccoid to ovoid	804, 868
Erythrobacter (Shiba and Simidu, 1982)	Marine	Orange	Rods	800, 869
Erythromicrobium (Yurkov et al., 1992)	Freshwater, thermal spring	Red-orange	Rods, branched	798, 832, 868
Erythromonas (Yurkov et al., 1997)	Freshwater, thermal spring	Orange-brown	Ovoid	800, 867
Hoeflea (Biebl et al., 2006)[2]	Marine	Colorless to pink	Rods	ND
Paracraurococcus (Saitoh et al., 1998)	Soil	Red	Coccoid	802, 856
Porphyrobacter (Fuerst et al., 1993)	Freshwater ponds, freshwater and brackish hotsprings, marine environments	Orange to red	Pleomorphic, rods	799-808, 867-870, (814 in *P. sanguineus*)
Roseateles (Suyama et al., 1999)	Freshwater, river	Pink	Rods	800, 870[1]

Table 1.1. Some determinative characteristics of genera containing APB (Continued).

Genus (described by)	Habitat and site of isolation	Color	Cell shape	*In vivo* Bchl peaks (nm)
Roseibacterium (Suzuki et al., 2006)	Sand	Pink	Rods	800, 879
Roseibium (Suzuki et al., 2000)	Coastal marine environments	Pink	Rods	803-805, 863-873[1]
Roseicyclus (Rathgeber et al., 2005)	Saline, meromictic lake	Pink-purple	Elongated rod to vibrioid	805-806, 870-871
Roseinatronobacter (Sorokin et al., 2000)	Soda lake	Pink	Lemon-shaped	803-805, 870[1]
Roseisalinus (Labrenz et al., 2005)	Saline, meromictic lake	Red	Rods	800-801, 870
Roseivivax (Suzuki et al., 1999a)	Saline lake	Pink	Rods	803-805, 871-873[1]
Roseobacter (Shiba, 1991)	Marine	Pink	Ovoid	806, 868
Roseococcus (Yurkov and Gorlenko, 1992)	Freshwater, thermal spring	Pink-red	Coccoid	806, 868
Roseovarius (Labrenz et al., 1999)	Saline, meromictic lake	Red, pink, light beige	Rods	799-802, 877-879
Rubrimonas (Suzuki et al., 1999b)	Saline lake	Pink	Short rods	806, 871[1]
Rubritepida (Alarico et al., 2002)	Freshwater, thermal spring	Red	Short rods	ND

Table 1.1. Some determinative characteristics of genera containing APB. (Continued)

Genus (described by)	Habitat and site of isolation	Color	Cell shape	*In vivo* Bchl peaks (nm)
Sandaracinobacter (Yurkov et al., 1997)	Freshwater, thermal spring	Yellow-orange	Thin, long rods	800, 867
Sandarakinorhabdus (Gich and Overmann, 2006)	Freshwater lake	Orange-red	rods	800, 837, 865
Staleya (Labrenz et al., 2000)	Saline, meromictic lake	Beige to yellowish-brown	Short rod	800-802, 861-865
Stappia (Kim et al., 2006)[2]	Tidal flat	ND	Club-shaped rods	ND
Thalassobacter (Macian et al., 2005)	Marine	Salmon-pink	Ovoid to irregular rods	ND

ND, not determined. Absorption spectra measured in: [1] membrane preparations. [2] The reference given corresponds to the description of the first phototroph included into the genus.

supralittoral zone on the Island of Texel in The Netherlands (Yurkov and Van Gemerden, 1993a; Yurkov et al., 1994b). More recently, *Stappia marina*, from a tidal flat in Korea, *Dinoroseobacter shibae* and *Hoeflea phototrophica*, from the North Sea, *Thalassobacter stenotrophicus,* from the Mediterranean (Macian et al., 2005), as well as several marine species of the genus *Porphyrobacter* have been described (Hiraishi et al., 2002; Yoon et al., 2004b; 2006). *Roseibacterium elongatum*, purified from sand at Shark Bay in Australia (Suzuki et al., 2006), can also be considered as a marine organism. Numerous reports of *Erythrobacter*-like and *Roseobacter*-like isolates from marine waters have surfaced, but as yet they have not been taxonomically identified (Kolber et al., 2000; 2001; Koblizek et al., 2003; Yurkov and Csotonyi, 2006).

The most unexpected discovery was the report of *Citromicrobium bathyomarinum* in the deep Pacific Ocean, a yellow pigmented APB exhibiting broad tolerance to salinity, temperature and pH. Several strains of *C. bathyomarinum* came from about 2000 m depth, in the vicinity of hydrothermal vent plume waters (Yurkov and Beatty, 1998b; Yurkov et al., 1999). Additional attempts have been made to recover this organism from the deep ocean away from hydrothermal vent areas, as well as surface waters (Yurkov and Beatty, 1998b; Kolber et al., 2001), but without success. Although infrared fast repetition rate (IRFRR) fluorometry showed large numbers of anoxygenic phototrophs at the surface (Kolber et al., 2000; Kolber et al., 2001) (see section 1.3.2), *C. bathyomarinum* is uniquely adapted to life in the deep ocean. This hypothesis is supported by the absence of 16S rDNA sequences related to *C. bathyomarinum* in surface samples from both neritic and oceanic systems (Beja et al., 2002).

Several strains from saline lakes along Australia's coasts (Shiba et al., 1991) have also been taxonomically described. *Rubrimonas cliftonensis* (Suzuki et al., 1999b), *Roseivivax halodurans* and *Roseivivax halotolerans*, (Suzuki et al., 1999a) are pink colored, rod shaped isolates from charophytes and epiphytes on stromatolites. *R. cliftonensis* exhibits an unusual LH complex II, with a single absorption peak at 806 nm (Shiba et al., 1991), previously known only in *Roseobacter* (Shiba, 1991; Yurkov and Beatty, 1998a). However a similar LH II has since been reported in strains from a meromictic lake in western Canada (Yurkova et al., 2002; Rathgeber et al., 2005), and may be reminiscent of the monomodal LH 4 of *Rhodopseudomonas palustris* (Hartigan et al., 2002; Evans et al., 2005). Both *Roseivivax* species show tremendous ability to survive in saline environments, growing at NaCl concentrations as high as 20%. *R. halodurans* is capable of growth in the absence of NaCl, indicating an adaptation to fluctuating levels of salinity as may be found in a saline lake (Hall and Northcote, 1986).

Roseovarius tolerans, *Staleya guttiformis* and *Roseisalinus antarcticus* (Labrenz et al., 1999; 2000; 2005) were purified from a water sample taken at Ekho Lake, a meromictic hypersaline and heliothermal lake in east Antarctica, remarkable for its high microbial morphological diversity, and for an almost complete absence of prokaryotic photosynthetic primary producers (Labrenz et al., 1999).

Several strains of *R. tolerans* induced BChl production only after six years of subculturing (Labrenz et al., 2000). Such unusual conversion in the laboratory indicates that unknown factors may influence the activation of photosynthesis genes, and suggests that some APB can be overlooked due to low or variable production of BChl.

Recently, APB were characterized from a hypersaline spring in Manitoba, a unique habitat with NaCl concentrations fluctuating between 6% and saturation. Not surprisingly these isolates were the most halophilic APB known to date, tolerating up to 26% NaCl, with optima as high as 18% (Csotonyi and Yurkov, 2006).

Due in part to industrial demand for alkaliphilic microorganisms, the search for new species has expanded to soda lakes (Jones et al., 1998), where a biogeochemical sulfur cycle is active and oxidation of reduced sulfur compounds is carried out by anaerobic phototrophs and aerobic chemotrophic sulfur oxidizers. Sorokin et al. (2000) reported an aerobic phototroph, *Roseinatronobacter thioxidans*, from a soda lake in the south-eastern part of Russian Siberia. The isolate is incapable of oxidizing sulfur compounds through anoxygenic photosynthesis, although, similar to *Erythromicrobium hydrolyticum* and *Roseococcus thiosulfatophilus* (Yurkov et al., 1994a) it is capable of aerobic heterotrophic oxidation of thiosulfate. This capability may represent an evolutionary transition between an anaerobic phototrophic sulfur based metabolism, and an aerobic heterotrophic lifestyle (Sorokin et al., 2000).

The first freshwater APB were collected from cyanobacterial mats located in hot springs near Baikal Lake in Russia (Yurkov and Gorlenko, 1990), and assigned to the genera *Sandaracinobacter*, *Erythromonas*, *Erythromicrobium* and *Roseococcus* (Yurkov and Gorlenko, 1992; 1993; Yurkov et al., 1992; 1994b; 1997). Freshwater hot springs continue to be a plentiful source of APB, and have in recent years yielded three genera. The acidophilic *Acidisphaera rubrifaciens* inhabits various acidic hot springs and acid mine drainage sites in Japan (Hiraishi et al., 2000). *Rubritepida flocculans*, from a hot spring in Hungary, is a moderate thermophile that produces BChl *a* when grown at 30°C,

but not at 50°C (Alarico et al., 2002). Apparently, temperature controls the expression of the photosynthetic apparatus in *Rubritepida*. *Porphyrobacter cryptus*, from a hot spring at Alcafache in central Portugal, has a growth temperature optimum of 50°C (Rainey et al., 2003).

The genus *Porphyrobacter* was published in 1993 to include BChl-containing aerobes, branching with *Erythrobacter longus* within the *Alphaproteobacteria*. *Porphyrobacter* differed from *Erythrobacter* in habitat, originating from a freshwater pond, and by an ultrastructural resemblance to the *Planctomycetales*, a tendency toward pleomorphism, replication by polar growth or budding and the production of multifibrillar stalk-like structures, as well as the absence of cytochrome (cyt) *c* oxidase and a lack of vitamin requirements (Fuerst et al., 1993). Currently, *Porphyrobacter* includes six species, from a wide variety of habitats: the type species *P. neustonesis,* the moderate thermophiles *P. tepidarius* (Hanada et al., 1997) and *P. cryptus* (Rainey et al., 2003), the budding *P. sanguineus* (Hiraishi et al., 2002) and the marine *P. donghaensis* and *P. dokdonensis* (Yoon et al., 2004b; 2006).

Despite the discovery of *P. neustonensis* more than a decade ago, few isolates of the APB originate from freshwater lakes or rivers. *Sanarakinorhabdus limnophila* was cultured using a high-throughput technique, from freshwater lakes in Germany (Gich and Overmann, 2006). *Roseateles depolymerans*, from the Hanamuro River in Japan, produces BChl *a* when grown on agar media containing polyhexamethylene carbonate, and is distinguished as the first APB phylogenetically placed within the β-subclass of the *Proteobacteria* (Suyama et al., 1999). Therefore the APB might be phylogenetically more diverse than previously thought. Of course, *R. depolymerans* requires further study,

including an in-depth examination of its ability/inability to carry out photosynthetic electron (e^-) transport in the presence/absence of oxygen, to ensure definitive APB features. It cannot be ruled out that this strain may behave differently from typical APB, and actually represents an interesting new sub-group.

The majority of APB known originate from aqueous environments. However, two interesting species, *Craurococcus roseus* and *Paracraurococcus ruber*, have been discovered in urban soils in Japan. They behave unusually having a tendency to form fragile cells in the absence of divalent cations in liquid media (Saitoh et al., 1998). Such behavior should be examined in close detail to determine whether or not this is a unique property. At present, very little is known about free living soil APB, although the legume-symbiote *Bradyrhizobium* has also been shown to produce BChl *a* (Fleischman and Kramer, 1998), and so this habitat presents an interesting new direction of study.

The most reliable method for identification and enumeration of APB is based on classical procedures, including the sometimes difficult isolation of pure cultures, an approach hindered by the apparent unculturability of many microorganisms (Jannasch and Jones, 1959), followed by analysis of photosynthetic pigments and time consuming physiological and biochemical tests (Yurkov and Beatty, 1998a; Yurkov, 2001).

In recent years, some new methods have been used to enhance our understanding of the diversity and ecological role of the APB. Sequencing of PCR-amplified 16S rDNA fragments has emerged as an important technique to evaluate genomic diversity in many natural environments. This approach has been used extensively, with DNA extracted directly from marine samples for the phylogenetic identification of microorganisms (Mullins et al., 1995).

Molecular techniques have indeed proven to be a powerful tool in the identification and enumeration of new phylogenetic types of bacteria. It appears that difficult to culture *Roseobacter* clade members comprise one of the major constituents of the world's oceans (Mullins et al., 1995). The weakness of the method lies in the lack of physiological data. All known APB (except *R. depolymerans*; see section 1.3.1) are distributed throughout the α-*Proteobacteria*, intermingled with the phototrophic purple non-sulfur bacteria and closely related chemotrophs (Rathgeber et al., 2004; Yurkov and Csotonyi, 2006). Clearly, due to the close proximity of most APB to non-phototrophs, (in many cases more than 98% 16S rDNA sequence similarity) phylogenetic data alone do not suffice as an identifying characteristic. Therefore, all marine isolates and extracted DNA fragments identified as belonging to *Roseobacter* sp., without supporting physiological and biochemical evidence (most notably the presence of BChl) must be treated tentatively; this is because such data may not indicate aerobic photoheterotrophs but instead also include chemotrophic *Roseobacter* relatives (Rathgeber et al., 2004).

Although much work has been done on habitats that harbor APB, little is yet known of their ecological role. As mentioned above, many isolates come from harsh conditions, including extremes of pH, temperature and salinity, but this may be a result of sampling bias, as work has focused on these sites in recent years. It is possible that phototrophy confers some advantage on APB over other heterotrophs when living under sub-optimal conditions (Yurkov and Csotonyi, 2003), although it seems more likely that APB might be equally abundant and important in less studied, non-extreme environments.

1.3.2. The importance of aerobic phototrophs in the ocean.

The discovery of *C. bathyomarinum*, in the lightless deep ocean (Yurkov and Beatty, 1998b; Yurkov et al., 1999), sparked immense curiosity in the role of APB in oceanic environments. In addition to the numerical importance of *Roseobacter* relatives in the marine community established by 16S rDNA sequences, remarkable biophysical results showed that APB-based photosynthesis was widespread in the open ocean (Kolber et al., 2000) and, subsequently, that APB may play an important role in oceanic carbon cycling (Kolber et al., 2001). Using a recently developed IRFRR fluorometer (Kolber et al., 1998), a fluorescence transient at 880 nm ascribed to anoxygenic bacterial light-driven e^- transport was detected (Kolber et al., 2000). IRFRR fluorometry of surface water taken from several Pacific and Atlantic Ocean sites detected a bacterial photosynthesis signal in all samples. The calculated proportion of anoxygenic phototrophs to phytoplankton was about 1 to 10%, increasing in oligotrophic waters (Kolber et al., 2000). Because the APB are capable of photoheterotrophy, they may have an advantage over strictly heterotrophic competitors under extreme nutrient depletion. Interestingly, IRFRR failed to detect fluorescence transients in the vicinity of deep ocean hydrothermal vent plumes, the isolation site of *C. bathyomarinum* (Yurkov et al., 1999). Although this may be due in part to the very low content of BChl per cell in this unusual species (Yurkov and Beatty, 1998b; Yurkov et al., 1999), it might also suggest that the abundance of APB in the deep ocean is much lower than at the surface.

Bulk BChl *a* at the Pacific Ocean surface measured by HPLC resulted in a maximum of about 4 ng per liter. The proportion of BChl *a* to chlorophyll (Chl) *a* was as high as 10% in oligotrophic waters, supported by IRFRR and direct counts of fluorescent

single cells (Kolber et al., 2001). Since that time, numerous additional endeavors have been undertaken to quantify APB. An independent sampling off the coast of southern California, by HPLC, yielded a significantly lower proportion of BChl *a* to Chl *a* of 0.7% in oligotrophic waters (Goericke, 2002), a level too low to support the contribution of bacterial photosynthesis proposed by Kolber et al. (2001). Thus, there appear to be significant differences in the concentrations of APB, depending on the locale and perhaps time of year. Several techniques based on infra-red epifluorescence (IREM) microscopy have been used to detect BChl-containing bacteria. These studies proposed wildly varying estimates of APB numbers, from 0.8 to 18% of the total bacterial population at the Mid Atlantic Bight (Cottrell et al., 2006), <1.5% at the North Pacific Gyre (Cottrell et al., 2006), 0.8 to 9.4% in the northwest Atlantic and Sargasso Sea (Sieracki et al., 2006), and 1.7% in the Eastern Pacific Ocean (Schwalbach and Fuhrman, 2005). Counting cells in marine waters is clearly problematic, as techniques do not adequately differentiate between APB and typical facultatively anaerobic phototrophic bacteria, and can even misidentify cyanobacteria (Yurkov and Csotonyi, 2006). Additionally, photosynthetic activity is greatly influenced by a variety of environmental factors. In all tested APB, BChl synthesis is strongly inhibited by even low levels of illumination, meaning that sampling times (day/night) or seasons can dramatically affect the amount of BChl in an active cell (Yurkov and Beatty., 1998a). Production of BChl can also be controlled by nutritional factors, with greater productivity generally occurring under oligotrophic conditions, as well as temperature, salinity and pH (Alarico et al., 2002; Koblizek et al., 2003; Rathgeber et al., 2004, Yurkov and Csotonyi, 2006). Because of this, it is

especially important to ensure that counting methods do not underestimate APB because of low BChl brought on by the environment.

Despite difficulties in quantification, the IRFRR data gave a clear indication of anoxygenic photosynthetic e^- transfer reactions at the surface of the open ocean, and made it obvious that photoheterotrophs may play an important role in carbon cycling. The ability to produce up to an estimated 20% of cellular energy requirements through photoheterotrophy results in a decreased requirement for heterotrophic oxidation of organic carbon to fulfill these energy needs, and in preservation of organic carbon (Kolber et al., 2001; Yurkov and Van Gemerden, 1993b). However, it is important to note that although APB were shown to be capable of low levels of light stimulated anapleurotic CO_2 incorporation (Kolber et al., 2001), they are not capable of autotrophic growth and therefore do not serve as primary photosynthetic producers of fixed carbon, but only as organic carbon metabolizers in nutrient cycling (Yurkov and Csotonyi, 2006).

The ecological function of *C. bathyomarinum* is still poorly understood. At present it has only been cultured from deep sites in the Pacific Ocean (Rathgeber et al., 2004; Yurkov and Csotonyi, 2006), and the purpose of its photosynthetic apparatus remains a mystery. Two major questions arise: 1) Is *C. bathyomarinum* endemic to hydrothermal vent plumes, or does it live throughout the water column? 2) Is its photosynthetic apparatus functional, in terms of light induced e^- transfer, and can it be used as an auxiliary source of energy? These questions clearly demonstrate that further research on the habitat and metabolism of *C. bathyomarinum* is essential to our understanding of this organism, and its role in the permanently dark deep ocean.

1.3.3. Issues in taxonomy.

At present much confusion exists within taxonomy. The scientific community has been split with regards to the respective roles of phylogeny and phenotype in bacterial systematics. On one hand is the belief that taxonomy should represent evolutionary relatedness among units, where all taxa form phylogenetically coherent clusters. This approach is hindered by the fact that molecular methods employed are subjective and 16S rRNA gene phylogenies are sometimes unable to resolve branching points with adequate statistical significance (Rainey et al., 2003). On the other hand, some believe that the role of taxonomy is to categorize microorganisms into groups of like organisms, based on unifying phenotypic characteristics. Although the International Committee on Systematic Bacteriology has stressed that phenotypic traits should remain important criteria in the delineation of taxa (Wayne et al., 1987; Murray et al., 1990; Stackebrandt et al., 2002), genus characteristic features are frequently ignored in the description of new species.

Because of the tendency to place phylogeny above phenotype, a growing number of non-phototrophic strains have been taxonomically positioned in genera of the APB. Examples include *Roseobacter algicola* (Lafay et al., 1995), *Roseobacter gallaeciensis* (Ruiz-Ponte et al., 1998), *Erythrobacter citreus* (Denner et al., 2002), *Erythrobacter flavus* (Yoon et al., 2003), *Erythrobacter aquimaris* (Yoon et al., 2004a), *Erythrobacter vulgaris* (Ivanova et al., 2005), *Erythrobacter luteolus* (Yoon et al., 2005a), *Erythrobacter gaetbuli*, *Erythrobacter seohaensis* (Yoon et al., 2005b), *Roseovarius crassostreae* (Boettcher et al., 2005) and *Roseovarius nubinhibens* (Gonzàlez et al., 2003). These species all lack BChl *a* incorporated into RC and LH complexes, but nevertheless they have been assigned to phototrophic genera primarily because of

phylogenetic (16S rDNA sequence) relatedness. Likewise, two species of APB, *Stappia marina* (Kim et al., 2006) and *Hoeflea phototrophica* (Biebl et al., 2006), have been classified into non-phototrophic genera.

Throughout this thesis I will take the stance that major phenotypic differences, such as the presence/absence of photosynthetic pigments, the ability to utilize a restricted mode of energy generation, morphology and physiology have always been and should remain important taxonomic markers. Furthermore, the rules of nomenclature require that the addition of a new species to an existing genus should correspond to the main genus distinctive properties. The purpose of the current bacterial taxonomic system is to group together organisms that exhibit similar characteristics, including morphological and physiological traits, habitats and modes of energy generation, to aid in the study of similar organisms. Therefore 16S rDNA phylogeny, although a valuable tool, cannot be used as a single or overriding determinative factor in taxonomy.

1.3.4. Photosynthetic pigments.

Pigment molecules are essential to the capture of light and transformation of its energy into a useable form. Like most purple phototrophic bacteria, APB possess BChl *a*, serving as the RC special pair (see section 1.3.5) and as the major antennae pigment. In contrast to anaerobic anoxygenic phototrophic bacteria (AnAP), APB are not known to possess BChl *b*, and accumulate relatively little BChl per cell (Yurkov and Beatty, 1998a) frequently containing less than $1/10^{th}$ that of purple non-sulfur bacteria (Yurkov and Beatty, 1998a).

A complex set of regulatory mechanisms is thought to influence production of BChl (Yurkov and Csotonyi, 2003; 2006; Rathgeber et al., 2004). Most notably synthesis is modulated by O_2 availability and is strongly repressed by light (Yurkov and Beatty, 1998a; Masuda et al., 1999; Suyama et al., 2002; Yurkov and Csotonyi, 2003; Rathgeber et al., 2005; Cooney et al., 2006). Under cycles of a light:dark regimen, several species have been observed to increase production of BChl relative to cultures maintained in constant darkness (Yurkov and Van Gemerden, 1993b; Biebl et al., 2006; Cooney et al., 2006). Other factors influencing pigment synthesis in some strains are stresses of temperature, pH and salinity (Rathgeber et al., 2004; Macian et al., 2005; Biebl et al., 2006), indicating that photosynthesis may impart an advantage to cells growing under sub-optimal conditions. In some species, anoxygenic photosynthesis under aerobic conditions is believed to function as a source of auxiliary energy when nutrients are limiting (Beatty, 2002), and it is not surprising that *R. depolymerans* was shown to induce BChl production upon sudden dilution of its carbon source (Suyama et al., 2002).

The second class of pigments found in APB is abundant carotenoids, which impart intense colors to cell cultures. In AnAP carotenoids can play three major roles: Contribute structurally to photosynthetic antenna complexes; Act as accessory LH pigments, allowing the use of light in the blue-green and yellow regions of the spectrum (Fraser et al., 2001) that is not absorbed by BChl; Confer protection of the photosynthetic apparatus from triplet oxygen formed under illuminated aerobic conditions when excited BChl reacts with O_2. Carotenoids have been shown to quench O_2 radicals and the excited BChl triplet itself. To act in photoprotection, they must be in direct contact with BChl to quench triplets effectively (Fraser et al., 2001), however most carotenoids of APB are not

associated with the photosynthetic apparatus and are distributed throughout the cell (Yurkov and Beatty, 1998a; Yurkov and Csotonyi, 2006). The composition is species specific and often includes carotenoids of unique chemical structure: 1) Acyclic carotenoids such as spirilloxanthin and spheroidenone, also found in AnAP; 2) The unique monocyclic bacteriorubixanthinal; 3) Bicyclic carotenoids such as β-carotene, rarely found in AnAP but distributed among oxygenic phototrophs; 4) Highly polar carotenoids such as erythroxanthin sulfate, which were first discovered in APB; 5) Highly polar C_{30} carotenoid glycosides, which are not found in other anoxygenic phototrophs (Harashima and Nakada, 1983; Takaichi et al., 1988; 1990; 1991; Noguchi et al., 1992; Yurkov et al., 1993; Yurkov and Beatty, 1998a). At present the role of these pigments is not well understood, possibly they serve as antioxidants, removing O_2 radicals of non-photosynthetic origin, or they act to filter high intensities of blue light, helping to minimize photodamage during periods of exposure to intense solar radiation (Yurkov and Beatty, 1998a; Yurkov and Csotonyi, 2006).

1.3.5. Photosynthesis in APB

Photosynthesis can be defined as a set of biological reactions that convert light energy into chemical energy, which can be stored in the form of ATP. Usually the process is coupled with the reduction of CO_2 to produce organic carbon for cellular building blocks. Most anoxygenic phototrophs are capable of photoautotrophy, however some will facultatively switch to a photoheterotrophic lifestyle under dark organic rich conditions (Dubbs and Tabita, 2004). The APB are an example of phototrophs that are incapable of autotrophic CO_2 fixation, do not produce ribulose bisphosphate carboxylase,

a key enzyme of the Calvin cycle (Yukov and Beatty, 1998a; Yurkov and Csotonyi, 2006), and like the gram-positive phototrophic heliobacteria, are therefore dependant on the presence of reduced organic carbon, even when using photosynthesis as a source of auxiliary energy.

Phototrophy can be divided into two separate and distinct types. In oxygenic phototrophy, H_2O serves as the e^- donor for the production of reducing power, and O_2 is evolved as a byproduct. It is found in the cyanobacteria and all eukaryotic phototrophs, and involves two different photosystems, similar to plants (Blankenship, 1992). In the second type, anoxygenic photosynthesis, found in all other phototrophic prokaryotes, a reduced compound other than H_2O serves as e^- donor, O_2 is not produced and only one photosystem is involved (Blankenship, 1992). The photosynthetic RC can be either of the ferredoxin type, as found in the green sulfur bacteria and phototrophic *Firmicutes* (heliobacteria), or it can be of the quinone type, found in the phototrophic *Proteobacteria* (Blankenship, 1992). Both oxygenic and anoxygenic photosystems can also function in a cyclic manner, where an external donor is not required (Brune, 1989; Blankenship, 1992).

The most well studied phototrophs of the *Proteobacteria* are the so-called purple bacteria and include the purple non-sulfur (α- and β-*Proteobacteria*) and the purple sulfur (γ-*Proteobacteria*) bacteria. The purple bacteria are AnAP in which photosynthetic e^- transfer occurs only under anaerobic conditions, and the production of photosynthetic units is generally stimulated by light and inhibited by oxygen. Two notable exceptions, *Rhodovulum sulfidophilum* (formerly *Rhodopseudomonas sulfidophila*) and *Rhodospirillum centenum* (homotypic synonym, *Rhodocista centenaria*) make BChl regardless of oxygen concentration (Hansen and Veldkamp, 1973; Nickens et al., 1996).

Photosynthesis involves two large trans-membrane complexes, the RC and the cyt bc_1 complex, and a number of cytoplasm soluble and membrane located e^- carriers which participate in cyclic e^- transfer, resulting in translocation of H^+ across the membrane and production of a proton motive force (Brune, 1989; Blankenship, 1992).

The photosynthetic apparatus of AnAP consists of several parts: The photochemical RC, made up of three structural protein subunits (PufL, M and H) (Lancaster and Michel, 1996; Stowell et al., 1997), four molecules of BChl *a*, two of which are known as the special pair of BChl and function as primary e^- donor (P), two bacteriopheophytins, two quinones (Q_A/Q_B), one non-heme high spin Fe^{2+} and carotenoids (Lancaster and Michel, 1996). Surrounding the RC is the LH I complex, made up of two subunits (PufA and B) (Masuda et al., 2000) and housing approximately 32 molecules of BChl *a* (Cogdell et al., 1996; 1999; Drews, 1996). The LH I absorbs light quanta and converts them to singlet excited states (excitation energy) that can be transferred to the RC. Together the RC and LH I form the core complex, essential for *in vivo* light driven e^- transport. Most AnAP also produce a secondary antenna structure, the LH II, which contains about 27 BChl molecules and aid in the collection of quanta (Cogdell et al., 1999). The structure of LH II is highly variable resulting in differences in absorbance spectra (Cogdell et al., 1996), and often expressional differences are observed in cultures grown under low-light intensities (Hartigan et al., 2002; Evans et al., 2005).

The LH I of APB closely resembles that of AnAP, in most cases giving absorbance maxima at about 870 nm (designated B870), although some LH I exhibit significant spectral shifting. Species of *Acidiphilium* produce a modified Bchl *a* incorporating an atom of Zn into the active site, in place of Mg found in other BChl.

Substitution of Zn has been proposed to stabilize the molecule at low pH, and leads to blue-shifting (864 nm) of LH I (Hiraishi and Shimada, 2001). This unique BChl can also be produced in small quantities by *Acidisphaera rubrifaciens* under laboratory conditions (Hiraishi et al., 2000). Other species exhibiting spectrally shifted LH I include *R. thiosulfatophilus* (B856) and *Roseovarius tolerans* (B879), likely due to structural factors within the pigment-protein complex (Yurkov et al., 1993; Labrenz et al., 1999).

The LH II of APB vary widely in expression and structure (Yurkov and Csotonyi, 2006), and show striking differences from LH II of purple AnAP. *Erythromicrobium* sp, *S. limnophila* and *P. dokdonensis* produce a B800-B830 (Yurkov and Beatty, 1998a; Gich and Overman, 2006; Yoon et al., 2006) and *P. donghaensis* produces B800-B814 (Yoon et al., 2004b), which are considerably blue-shifted compared to LH II typically found in AnAP. Species of the genera *Roseobacter*, *Rubrimonas* and *Roseicyclus* (Shiba, 1991; Suzuki et al., 1999b; Rathgeber et al., 2005) possess an interesting monomodal LH II with a single absorbance peak at around 805-806 nm, similar to that found in the AnAP *R. palustris* (B800) grown under low light intensities (Hartigan et al., 2002; Evans et al., 2005). These complexes are able to harvest more energetic wavelengths of light and may be an adaptation of APB used to growing in well oxygenated surface habitats, where higher energy radiation is available (Yurkov and Csotonyi, 2006).

Photosynthetic e^- transfer requires, in addition to the core complex and optional LH II, a second major transmembrane protein, the cyt bc_1 complex, which functions in both the photosynthetic and respiratory e^- transport chains (Crofts and Wright, 1983). Energy transduction occurs when excitation energy is transferred from LH pigments to the special pair, resulting in a singlet excited state BChl that acts as an extremely strong

reductant and transfers e^- through bacteriopheophytin to the Q_A, touching off a cyclic e^- transport cascade. The Q_A then passes e^- to quinones located in the membrane. The photooxidized P (P^+) can be reduced one of two ways. In the majority of AnAP, e^- are very quickly donated from a RC bound tetrahemic cyt *c*, which is then re-reduced by a periplasm soluble cyt (usually cyt c_2). In phototrophs lacking a RC-bound cyt, reduction of P^+ occurs more slowly and is mediated directly by a soluble cyt (Meyer and Cusanovich, 2003). In either case, the soluble cyt is then re-reduced by the cyt bc_1 complex. The reduced membrane quinone pool delivers e^- to the cyt bc_1 completing the cycle, and in so doing mediates the translocation of H^+ across the membrane, establishing a H^+ gradient. This H^+ motive force can be used to power an F_1/F_0 ATPase for the conservation of energy in the usable form of ATP (Okamura et al., 2000). Such photosynthetic e^- transfer is functional only under anaerobic conditions, and accordingly production of BChl and thus the photosynthetic apparatus is halted in the presence of O_2 (Drews and Oelze, 1981).

Photosynthesis in APB closely resembles that of AnAP, however, it is functional only under aerobic conditions, and inoperative under anaerobiosis (Okamura et al., 1985; Garcia et al., 1994; Yurkov et al., 1995; Yurkov and Beatty, 1998a; Rathgeber et al., 2004; Yurkov and Csotonyi, 2006), in agreement with their inability to grow phototrophically under anaerobic conditions. Photosynthetic e^- transfer in APB, like AnAP, can be distinguished into two separate types based on the mode of the P^+ re-reduction. The first involves a cyt *c* intimately bound to the RC, allowing for very fast re-reduction of the P^+. This type of e^- transfer has been observed in *Erythromonas ursincola*, *Sandaracinobacter sibiricus*, *R. thiosulfatophilus* and *R. denitrificans* (Garcia, et al.,

1994; Yurkov et al., 1998b, Yurkov and Beatty, 1998a). In the second, found in all tested species of α-4-*Proteobacteria*, no RC-bound cyt is present and P^+ is re-reduced through transfer of an e^- directly from a soluble cyt *c* (Yurkov et al., 1998b). In *E. litoralis*, which also belongs to the α-4 subclass, oxidation of the soluble cyt *c* was relatively slow, further differentiating its photochemistry from other APB (Yurkov et al., 1998b).

It is thought that the anaerobic inactivity of the photochemical e^- transport in APB is due to the relatively high redox midpoint potential of the Q_A, reported to range from +5 to +150 mV (Yurkov and Beatty, 1998a), much higher than the negative values commonly observed in purple non-sulfur phototrophs (Candela et al., 2001). Thus, under anaerobic conditions, Q_A would be reduced and incapable of accepting an e^- and participating in e^- transport (Yurkov et al., 1998b; Yurkov and Beatty, 1998a; Rathgeber et al., 2004). Alternatively, the redox state of cyt in the e^- transport chain may be responsible for the lack of anaerobic photochemistry (Garcia et al., 1994; Schwarze et al., 2000). Under reduced conditions, the low potential hemes of the *R. denitrificans* RC-bound tetraheme cyt were capable of re-reducing P^+, but the hemes were not re-reduced by the soluble cyt *c*, thus halting e^- transfer reactions (Schwarze et al., 2000).

A complexity relating to the role of oxygen in the photosynthetic e^- transport of *R. denitrificans* comes from a study of the lipid-soluble quinone pool. *R. denitrificans* lacks a quinol oxidase pathway as is found in purple non-sulfur phototrophs such as *Rhodobacter capsulatus*. This quinol oxidase pathway is thought to help maintain the proper redox state of the quinone pool when oxygen is present. Because of the absence of a quinol oxidase, *R. denitrificans* may rely solely on e^- transfer from quinols to the cyt bc_1

complex and through a soluble cyt *c* and cyt *c* oxidase, to maintain a quinone redox state needed for aerobic phototrophic growth (Candela et al., 2001).

The regulation of photosynthesis gene expression in the APB appears to be different from AnAP, although both groups respond to the same environmental factors. For example, BChl synthesis is repressed by even low levels of illumination in the APB, whereas in AnAP, BChl synthesis is inhibited only by high light intensities. Production of the photosynthetic apparatus in APB also seems to be modulated by nutrient availability, as well as by factors such as pH, salinity and temperature (Yurkov and Beatty, 1998a; Hiraishi and Shimada, 2001; Rathgeber et al., 2004). Interestingly, environmental factors that might be described as extreme, such as extremely low pH in the case of *Acidiphilium* species (Hiraishi and Shimada, 2001), or high salinity in the case of strains isolated from a saline lake (Rathgeber et al., 2004), tend to result in an increased production of the photosynthetic apparatus, suggesting that an auxiliary aerobic phototrophic metabolism is of advantage to these bacteria living under sub-optimal conditions.

Components of photosynthesis in *R. denitrificans* and other APB are believed to be encoded on a *puf* operon, similar to the 45 kb superoperon of purple bacteria (Liebetanz et al., 1991; Klug, 1993; Nishimura et al., 1999; Yurkov and Csotonyi, 2006), although genes encoding RC polypeptides have been shown to reside on linear plasmids in *R. litoralis* and *Staleya guttiformis* (Pradella et al., 2004). At present very little is known about the molecular details of APB photosynthesis gene regulation. The photosynthesis regulatory gene cluster in *R. denitrificans* has been identified (Nishimura et al., 1999) and its organization is similar to that found in *Rhodobacter sphaeroides* and

R. capsulatus, although the regulation of the *puf* operon and pigment production differs with respect to the effect of oxygen and light (Nishimura et al., 1999).

In AnAP oxygen-dependant transcription of the *puf* operon is controlled by a two component regulatory system, consisting of a sensor kinase (RegB or PrrB) and a response regulator (RegA or PrrA) (Gregor and Klug, 1999; Oh and Kaplan, 2001). RegB undergoes autophosphorylation in response to an oxygen-dependant signal and in turn phosphorylates RegA, however, RegB appears to have no obvious oxygen sensing domain, and therefore may depend on a redox sensing protein (Gregor and Klug, 1999). Similar *RegA/RegB* genes are present in *R. denitrificans*, and exhibit a high level of sequence similarity to *Rhodobacter* homologues. Both RegA and RegB mutants of *R. capsulatus* complemented with the *RegA* or *RegB* genes from *R. denitrificans* produced pigments in response to oxygen deprivation (Masuda et al., 1999), giving little clue as to why *R. denitrificans* is able to produce these pigments under aerobic conditions. Like its homologues, the RegB of *R. denitrificans* does not have an obvious oxygen-binding domain (Nishimura et al., 1999), consistent with the hypothesis that an oxygen sensing protein may be required to signal autophosphorylation of RegB (Oh and Kaplan, 2001). If this hypothetical protein is absent or non-functional in *R. denitrificans*, it could help to explain the ability of APB to produce photopigments aerobically.

Other purple bacterial regulatory factors, such as CrtJ/PpsR and AppA (Masuda and Bauer, 2002), have not yet been found in the APB. In this respect, the genome sequence of a representative APB would be of great help to identify putative oxygen- and light-responsive regulatory proteins. The entire genome of *R. denitrificans* has recently been analyzed (Swingley et al., 2007).

Currently, anoxygenic phototrophic bacteria fall into two groups: conventional AnAP that grow robustly under illuminated anaerobic conditions, and APB that photosynthesize only in the presence of oxygen. The evolution of the photosynthetic apparatus, from anaerobic to aerobically active complex, remains unexplained. It has been suggested that APB may have evolved to fill a specific niche (Beatty, 2002), or that lateral transfer of photosynthesis genes may have occurred, giving rise to APB by "accident". The peculiarities of the photosynthetic apparatus are not yet fully understood, and it is still not known if there exists an intermediate evolutionary stage between AnAP and APB, an organism capable of photosynthesis independent of oxygen concentrations. Further study of photosynthesis in new and diverse species is essential to solve these dilemmas.

1.4. Heavy metalloid reducing bacteria.

1.4.1. Ecological perspectives.

Heavy metalloids, or semi-metals, are elements that exhibit some properties of metals, such as metallic luster and the inability to form monatomic anions, and some properties of nonmetals such as formation of covalent crystals and semi-conductance (Yurkov and Csotonyi, 2003). Toxicity of metalloid oxyanions has been attributed to strong oxidizing activity, producing highly reactive free radicals, which may cause oxidative stress and interfere with cellular processes (Summers and Jacoby, 1977). While a number of metalloids are toxic, this thesis will focus on tellurium (Te) and selenium

(Se), which occur in a variety of oxidized ionic forms, including tellurite (TeO_3^{2-}), tellurate (TeO_4^{2-}), selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}).

Relatively rare in the Earth's crust, Te is primarily associated with deposits of metals and sulfur (Eilu, 2001) and elevated levels of TeO_3^{2-} can be found in waste discharge from mining operations and in gold mine tailings (Wray, 1998). Te is not a biologically essential element and TeO_3^{2-} concentrations as low as 1 μg/ml can be toxic to most microorganisms (Summers and Jacoby, 1977). In fact, tellurite has been employed as an antimicrobial growth agent and was often used as a remedy for bacterial infections before the development of antibiotics (Taylor, 1999). Reduction of TeO_3^{2-} by bacteria results in the accumulation of black elemental Te leading to black colonies on agar plates and black cultures in liquid media (Moore and Kaplan, 1992; Taylor et al., 1988).

Se on the other hand is biologically essential, required for the amino acid selenocysteine (Stadtman, 1996), but is toxic at high concentrations (Lauchli, 1993) and in its oxidized states (Moore and Kaplan, 1992). Some bacteria can reduce SeO_3^{2-} to the less toxic elemental form, accumulating amorphous Se, which gives colonies and liquid cultures a bright red color (Moore and Kaplan, 1992). Additionally SeO_3^{2-} may also serve as the terminal e^- acceptor for anaerobic respiration in several species (*Thauera selenatis*, *Sulfospirillum barnesii*, *Bacillus arsenicoselenatis*, and *Bacillus selenitireducens*) (Macy et al., 1993; Switzer-Blum et al., 1998).

A wide variety of microorganisms are known to reduce metalloid oxyanions, although only few have been discovered exhibiting high level resistance (> 1000 μg/ml). Bacteria capable of resisting low levels (< 50 μg/ml) include the gram positive

Corynebacterium diptheriae, *Streptococcus faecalis* and *Staphylococcus aureus*, and gram negative *Alcaligenes faecalis*, *Alcaligenes denitrificans*, *Pseudomonas mendocina*, as well as *E. coli* and other *Enterobacteriacea* (Walter and Taylor, 1992; Rajwade and Paknikar, 2003). Those shown to reduce high levels of TeO_3^{2-} include the AnAP *R. sphaeroides* and *R. capsulatus*, the APB *E. hydrolyticum*, *Erythromicrobium ramosum*, *Erythromicrobium ezovicum*, *E. ursincola*. *R. thiosulfatophilus*, *S. sibiricus*, *C. bathyomarinum*, marine non-phototrophs of the genus *Pseudoaltermonas*, the facultative anaerobe *Shewanella oneidensis*, the thermophilic bacterium *Thermus thermophilus* and the *Archaea Natronococcus occultus*, *Natronobacterium magadii* and *Natronobacterium gregoryi* (Moore and Kaplan, 1992; Borghese et al., 2004; Yurkov et al., 1996; Rathgeber et al., 2006; Klonowska et al., 2005; Pearion and Jablonski, 1999). High level resistance and reduction of Se oxyanions is more widespread (Yurkov and Csotonyi, 2003) and occurs in most of the above organisms as well as *T. selenatis*, *S. barnesii*, *B. arsenicoselanatis*, *B. selenitireducens*, *Selenihalanaerobacter schriftii*, *Ralstonia metallidurans* and the *Archaea Pyrobaculum arsenaticum* and *Pyrobaculum aerophilum* (Macy et al., 1993; Switzer-Blum et al., 1998; Stolz and Oremland, 1999; Sarret et al., 2005).

Due to the scarcity of oxidized Te in naturally occurring environments, the ecological role of TeO_3^{2-} reduction is not well understood. It is believed that most transformations occur primarily for the purpose of detoxification, and in some cases reduction may be an unintended secondary function of enzymes involved in other metabolic pathways (see section 1.4.2). Recently bacteria that are capable of dissimilative reduction using TeO_3^{2-} as an e^- acceptor in anaerobic respiration were discovered

(Csotonyi et al., 2006), and it remains to be seen how widespread is this ability. Aerobic and anaerobic reduction of the Se oxyanions also occurs in cells for the purpose of detoxification, although dissimilative use of SeO_3^{2-} is well documented (Macy et al., 1993; Switzer-Blum et al., 1998).

The AnAP *R. sphaeroides* and *Rhodospirillum rubrum* have been suggested to use both TeO_3^{2-} and SeO_3^{2-} as a sink for excess e^- generated during photosynthetic growth (Moore and Kaplan, 1992), although these ions are not used as terminal e^- acceptors (Kessi et al., 1999), demonstrating the variety of reasons microorganisms might choose to reduce toxic metalloids.

1.4.2. Mechanisms of metalloid reduction.

Several strategies have been proposed to account for resistance to Te and Se compounds in microorganisms: reduced uptake of the metalloid; ejection of the toxin using ATP dependent efflux pumps and proton motive force dependent antiporters, and alteration of redox state leading to detoxification (Silver and Phung, 1996). In the case of oxyanions, reduction leads to the accumulation of less toxic elemental metalloids.

The reduction of TeO_3^{2-} occurs by several different mechanisms. In aerobically grown cultures of *R. sphaeroides*, components of the e^- transport chain, cyt bc_1 complex and a soluble cyt c_2 are involved (Moore and Kaplan, 1992). However, under anaerobic conditions, a membrane bound nitrate reductase seems to be responsible for TeO_3^{2-} reduction (Sabaty et al., 2001). Nitrate reductases have also been implicated in the low level of tellurite resistance in anaerobically grown *E. coli* (Avazeri et al., 1997). In other gram negative bacteria, the aerobic respiratory chain is involved, evidenced by the

accumulation of elemental Te at the position of terminal oxidases in the cytoplasmic membrane (Trutko et al., 2000). Other proteins of TeO_3^{2-} reduction resemble enzymes required for sulfate metabolism (Alonso et al., 2000), quenching of toxic metabolites (Dunn et al., 1999) and heat shock response (Karls et al., 1998). Tellurite resistance determinants can be encoded on plasmids in some Gram negative bacteria (Summers and Jacoby, 1977; Suzina et al., 1995; Taylor et al., 1988; Walter and Taylor, 1989) and in others, on the chromosome (Liu et al., 2000), but there is some evidence that chromosomal incorporation of plasmid located determinants may occur (Taylor et al., 2002). Although poorly understood, it appears that the primary mechanisms of TeO_3^{2-} reduction arise from the non-specific secondary action of enzymes involved in other key metabolic pathways. This may be due to the relative scarcity of Te in nature, as most bacteria do not require independent means of detoxifying TeO_3^{2-}. It will be interesting to see how the mechanism of reduction differs in strains capable of dissimilative TeO_3^{2-} reduction (Csotonyi et al., 2006), however, this has not yet been determined.

The reduction of Se oxyanions, SeO_3^{2-} and SeO_4^{2-}, can occur by similar means. In several Gram negative bacteria SeO_4^{2-}, like TeO_3^{2-}, is converted to its elemental form through the non-specific action of nitrate reductases (Sabaty et al., 2001). Many bacteria are capable of assimilating oxidized Se into organic molecules for the production of selenocysteine, including *R. metallireducens*, which reduces SeO_3^{2-} to Se via an organic intermediate (Sarret et al., 2005). Similarly, an organoselenium intermediate was observed during reduction of SeO_4^{2-} in *R. sphaeroides* and *Halomonas* sp. (Van Fleet-Stalder et al., 2000; de Souza et al., 2001). In contrast, *R. metallireducens* is only able to reduce small quantities of SeO_4^{2-}, suggesting that pathways of SeO_3^{2-} and SeO_4^{2-}

reduction may be different. This agrees with results found for *E. coli*, in which impairment of SeO_4^{2-} reduction by transposon mutagenesis did not affect SeO_3^{2-} metabolism (Bebien et al., 2002). In contrast to TeO_3^{2-} reduction, the genes involved in aerobic SeO_3^{2-} reduction have not yet been identified and the exact mechanisms are still relatively unknown. Anaerobic respiration of SeO_3^{2-} is believed to function similar to nitrate reduction and may involve the same enzyme in *S. barnesii* (Oremland et al., 1999), whereas the reductase of *T. selanatis* is selenate specific, but still bears structural resemblance to nitrate reductase (McEwan et al., 2002).

In addition to accumulation of elemental forms, both TeO_3^{2-} and SeO_3^{2-} may be reduced via methylation by some bacteria as a detoxification strategy, resulting in volatile compounds which escape to the atmosphere (Chasteen and Bentley, 2003). Methylation has been observed in *Pseudomonas aeruginosa*, *R. capsulatus* and *Rhodocyclus tenuis* (Trutko et al., 2000; Van Fleet-Stalder and Chasteen, 1998). In the case of methylated Te, the volatile waste-products can be even more toxic than the original oxyanions and may pose a significant threat to ecosystems (Yurkov and Csotonyi, 2003).

1.4.3. Industrial significance.

In their pure elemental form, Te and Se are commercially valuable semi-metals, with widely fluctuating prices due to inconsistencies in production. They are commonly used as additives to steel, brighteners in electroplating baths, colorants in the production of glass, as catalysts in petroleum cracking and other chemical reactions, as semiconductors in photovoltaic cells and as toners in photography (Rajwade and Paknikar, 2003). Increased use has led to contamination of industrial discharge by toxic

metalloid compounds, and disposal of metalloid wastes is a growing environmental concern. Additionally, enriched levels of metalloids can remain after conventional metallurgy, leading to pollution of surrounding ecosystems (Wray, 1998). Because most methods of removing metalloids are expensive and inefficient, recent interest has turned to the use of biological reductions to remediate metalloid contaminated areas (White et al., 1997). Despite the discovery of many TeO_3^{2-} and SeO_3^{2-} reducing strains, and much interest in the matter, little has yet been done to effectively utilize these processes for bioremediation.

Preliminary results are encouraging. A bioreactor using *Pseudomonas mendocina* grown aerobically on sucrose and $(NH_4)_2HPO_4$ allowed reduction of 10 μg/ml K_2TeO_3 with 99.8% efficiency after 3 days of batch culture, and could then be operated in a continuous mode (Rajwade and Paknikar, 2003). Although the addition of chemically pure organic substrates to the bioreactor may be expensive, it has been suggested that sucrose could be replaced with low cost molasses for large scale use. Similar batch and packed-bed, continuous flow bioreactors were used to evaluate SeO_3^{2-} reduction by a microbial consortium, enriched from oil refinery waste. Reduction of the metalloid proceeded under denitrifying conditions as long as nitrite did not accumulate in the growth medium (Viamajala et al., 2006), likely because SeO_3^{2-} reduction depends on the non-specific activity of enzymes involved in dissimilative NO_3^- metabolism. Although difficult to remove from solution because of its small particle size, Se could be effectively extracted from cells that produce intracellular accumulations (Viamajala et al., 2006). Extraction is an important factor in bioremediation of contaminated soils as Se can be quickly re-oxidized under appropriate conditions (Masscheleyn and Patrick, 1993).

Biohydrometallurgy is a promising technology for obtaining valuable metals from low grade ores or industrial wastes. It has been suggested that Te extraction from sites polluted with TeO_3^{2-} could be used in an industrial recovery of this valuable semi-metal (Yurkov and Csotonyi, 2003). The advantages of biological reduction over conventional recovery are low energy costs (Bombacher et al., 1997), providing economical growth conditions can be found. Although many metal extractions work well on a laboratory scale, effective biohydrometallurgy will require that these processes can be increased to commercial scale applications (Bombacher et al., 1997).

Clearly, bioremediation and biohydrometallurgy, involving the extraction and accumulation of elemental semi-metals from contaminated environments are attractive options for detoxifying industrial wastes. Further development will depend on the discovery of microorganisms and enzymes capable of efficiently processing large quantities of metalloids under a variety of controlled and uncontrolled parameters. For this reason, the search for new strains capable of transforming metalloids is paramount. As many industrial processes rely on enzymes capable of functioning under harsh conditions, extreme environments, such as deep ocean hydrotherms should present an excellent source for new microorganisms capable of reducing TeO_3^{2-} and SeO_3^{2-}.

1.5. Aims of the thesis.

1.5.1. Characterization of new strains isolated from the meromictic Mahoney Lake (Chapter 2).

Although unknown prior to 1979, APB have recently been found to inhabit a plethora of ecosystems (see section 1.3.1). However, our understanding of the true distribution of APB is still in its infancy, and undoubtedly many habitats harboring APB have yet to be discovered. Characterization of strains from different locations will allow us to appreciate the myriad roles APB play in nature. Additionally, recovery of new strains will bring to light interesting variations in pigment composition and photosynthetic apparatus organization (see section 1.3.4, 1.3.5) and will enrich our knowledge of the complex evolution of phototrophy.

In this thesis thirty-seven isolates of APB and unusual AnAP from Mahoney Lake are characterized in terms of morphology, physiology, biochemistry and phylogeny in order to discover interesting new extremotolerant strains, upon which further investigations of APB can be undertaken.

1.5.2. Evaluation of the potential to resist and reduce high concentrations of K_2TeO_3 and Na_2SeO_3 (Chapter 3).

Much interest currently exists with regards to the use of extremotolerant and extremophilic organisms as agents of bioremediation of industrial wastes. Additionally, biohydrometallurgy is an attractive option for economical extraction of valuable elements from polluted sites or low grade ores. Biological reductions of TeO_3^{2-} and SeO_3^{2-} are

viable options to carry out these processes. As an ideal candidate organism has not yet emerged for bioremediation, an extensive search is mandated for microorganisms that can resist and reduce the high levels of metalloids needed to fulfill the goal. For this reason the ability to resist and reduce TeO_3^{2-} and SeO_3^{2-} in ten isolates recovered from the vicinity of black smoker vents, was evaluated.

1.5.3. Description of new taxa (Chapter 4-6).

The taxonomic description of microorganisms is paramount to the study of microbiology. It allows us to arrange them in functionally meaningful groups, and helps us to understand their genetic relationships.

While an exhaustive description of all strains presented in the thesis is beyond its current scope, it was important to have taxonomic recognition of the strains upon which most of the work has been based. For this reason, proposals have been presented for new species of APB and heavy metalloid reducing bacteria.

1.5.4. Search for APB at deep ocean hydrothermal vents and throughout the water column of the Pacific Ocean (Chapter 7).

C. bathyomarinum is the first phototroph isolated from the permanently dark deep ocean (see section 1.3.2). Although ubiquitous in many extreme settings (Yurkov and Csotonyi, 2003), the discovery of APB in this environment in particular was met with many questions. The major two: Is *C. bathyomarinum* endemic to hydrothermal vent plumes, or does it live throughout the water column? Is its photosynthetic apparatus

functional, in terms of light induced e^- transfer, and can it be used as an auxiliary source of energy?

This thesis attempts to provide answers by investigating the vertical distribution of APB at five sites in the Pacific Ocean. Furthermore, convincing data demonstrate that the pigment protein complexes are capable of light induced e^- transfer and thus *C. bathyomarinum* is photosynthetically competent and should be able to supplement its energy needs when light is available.

1.5.5. Investigation of the photosynthetic apparatus of recently isolated APB (Chapter 8).

Results in several laboratories have confirmed that the APB photosynthetic apparatus is active under oxidized conditions, and lack of photochemistry under reduced conditions is consistent with their inability to grow anaerobically by photosynthesis. However questions pertaining to the peculiarities of light induced e^- flow in this group remain (see section 1.3.5). Is photosynthesis governed by the redox potential of Q_A? Is there really a marked discontinuity between APB and AnAP in terms of O_2's effect on photosynthesis, or is it possible that intermediate organisms can be found, in which phototrophy occurs under both aerobic and anaerobic conditions? In an attempt to answer these questions, two strains recovered from Mahoney Lake were chosen for an in depth study of the photosynthetic apparatus organization and e^- transfer.

1.6. References

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Chapter 2.

Diversity, distribution and physiology of the aerobic phototrophic bacteria in the mixolimnion of a meromictic lake.

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The material presented in Chapter 2 is derived equally from experiments completed by Natalia Yurkova and Christopher Rathgeber. Coauthors contributing to this paper were Jolantha Swiderski and Erko Stackebrandt, who performed the phylogenetic analysis; and Vladimir Yurkov, who guided the project. This research was initiated in the lab of J. Thomas Beatty.

2.1. Abstract.

The population of anoxygenic phototrophic bacteria in the aerobic zone of the meromictic Mahoney Lake was investigated using classical microbiological methods. This bacterial community was found to be very rich and diverse. Thirty one new strains of the obligately aerobic phototrophic bacteria, and two new purple nonsulfur strains, were isolated in pure laboratory cultures and preliminarily characterized. The isolates contain a variety of carotenoids, bacteriochlorophyll *a* incorporated into pigment protein complexes, and are morphologically and physiologically diverse. These properties indicate a diversity of adaptations to the stratified environments of this meromictic lake. Phylogenetically all isolated strains belong to the α-subclass of *Proteobacteria*.

2.2. Introduction.

Mahoney Lake is a meromictic saline lake, without an outflow, located near Penticton in the dry region of south central British Columbia. Meromictic lakes contain bottom waters (the monolimnion) that do not mix with the surface waters (the mixolimnion), separated by a chemocline (Wetzel, 1975). There is little seasonal change in the location of the chemocline in terms of salinity. However, the seasonal depth of the chemocline can vary between 0.5 to 0.75 m dependant on the level of evaporation. Usually the top of the chemocline and the loss of oxygen in the vertical profile is defined in the 8.0 to 8.5 m zone. In Mahoney Lake meromixis is maintained by a sharp chemical discontinuity at the chemocline. Through the water column, Na^+, Ca^{2+}, Mg^{2+} and SO_4^{2-} are the major inorganic ions in the lake water (Northcote and Halsey, 1969), attributed to the composition of lavas of the Marron formation in the watershed (Northcote and Hall,

1983), and so Mahoney Lake has been classified as a mainly sodium sulfate lake (Hall and Northcote, 1986).

Previous microbiological investigations of Mahoney Lake focused on anaerobic anoxygenic phototrophs and revealed an extremely dense population of the purple sulfur bacterium *Amoebobacter purpureus* in the chemocline of the lake (Overman et al., 1991; 1996). The enormous *A. purpureus* population was proposed to be the major route for transfer of phosphorus, by periodic upwelling events, from the monolimnion for the growth of heterotrophic bacteria in the mixolimnion of Mahoney Lake (Overman et al., 1991). Strains of *Rhodobacter capsulatus* (a purple nonsulfur bacterium), *Thiocapsa roseopersicina* (a purple sulfur bacterium), *Chloroherpeton thalasium* and *Prosthecochloris aestuarii* (green sulfur bacteria) were also isolated from the chemocline at about 7 m depth (Overman et al., 1996). Additionally, the cyanobacterium *Gloeocapsa* sp. has been found at depths up to 5 m (Northcote and Hall, 1983).

Our goal was to investigate the microbial population of the Mahoney Lake mixolimnion with focus placed on a relatively newly described physiological group of aerobic bacteria, the APB. Aerobic phototrophs have been found in different locations including the extreme environments of acidic mine drainage waters, hot temperature springs and deep ocean hydrothermal vent plumes (Yurkov and Beatty, 1998a). The novel aspect of this increasingly large group of bacteria that contain BChl *a* and carotenoid pigments is the inability to use BChl for anaerobic photosynthetic growth. The APB carry out limited anoxygenic photosynthesis under aerobic conditions, but light cannot be used as the sole source of energy and none of the aerobic phototrophic species has yet been shown to grow autotrophically. Most species are strict aerobes, and growth

is dependent on organic substrates as the main source of carbon and energy (Yurkov and Beatty, 1998a). At present APB are taxonomically classified into 15 different genera that include marine, freshwater and soil bacteria. A better understanding of the evolutionary origin and diversity of the physiological properties of this group depends on continued study of the existing species and isolation of new strains. In this paper we report the results of an investigation of new phototrophic bacteria isolated from different depths of the Mahoney Lake mixolimnion.

2.3. Materials and methods.

2.3.1. Collection of samples.

Samples were collected on October 3, 1997 from surface, 3 m and 5 m depths using a three liter van Dorn bottle over the deepest site of the lake. Samples were immediately transferred into sterile 500 ml screw-cap bottles and placed in dark storage at 4°C.

2.3.2. Enumeration of bacteria.

After return to the laboratory, decimal dilutions of the samples were prepared and 0.1 ml of each dilution was spread in duplicate on 2% agar plates of the following media.

Medium N1 contains (g/l): KH_2PO_4, 0.3; $MgSO_4$, 2.0; NH_4Cl, 0.3; KCl, 0.3; $CaCl_2 \cdot 2H_2O$, 0.05; Na_2SO_4, 15.0; $NaHCO_3$, 0.5; Na acetate, 1.0; Na malate, 1.0; yeast extract, 1.0; peptone, 0.5.

Medium N2: same composition as Medium N1 except that NaCl (15.0 g/l) is substituted for Na_2SO_4, and Na succinate (1.0 g/l) is substituted for Na malate.

Medium N3: same composition as Medium N1, but without Na_2SO_4.

Medium N4: same composition as Medium N1, with the concentration of Na_2SO_4 increased to 50.0 g/l.

All the media were adjusted to pH 7.8-8.0 and supplemented with a mixture of vitamins (per 1 of medium: 20 µg of vitamin B_{12}; 200 µg of nicotinic acid; 80 µg of biotin; and 400 µg of thiamin), and 2.0 ml per 1 of a trace element solution (Drews, 1983).

Plates were incubated aerobically at 30°C in the dark, and colonies were counted after 8 days. Pigmented colonies were resuspended in liquid media of identical composition and pure cultures were obtained by repeated plating.

The ability for anaerobic photosynthetic growth was tested in screw-cap test tubes completely filled with media for purple sulfur or nonsulfur bacteria (Imhoff, 1988), as well as the medium of isolation.

2.3.3. Spectral analysis.

Strains that formed pigmented colonies were grown under aerobic conditions at 32°C in rotating test tubes. After centrifugation (1-2 min at 15K rpm), pellets were re-suspended in 125 µl of 10 mM Tris-HCl buffer (pH 7.8) and mixed with 375 µl of a 30% BSA solution (to reduce light-scattering). Absorbance spectra were recorded between 350 and 1100 nm at room temperature with a Hitachi U2000 spectrophotometer.

2.3.4. Electron microscopy.

Cells from log phase cultures grown aerobically in liquid medium were negatively stained with 1.0% aqueous uranyl acetate. For thin sections, the bacteria were embedded in Epon after fixation with 1.0% glutaraldehyde and 1.0% osmium tetroxide as described (Kellenberger et al., 1958).

2.3.5. Physiology.

Physiological and biochemical tests were performed as previously described (Yurkov and Van Gemerden, 1993; Yurkov et al., 1994)

2.3.6. 16S rDNA-based phylogenetic analysis.

Extraction of genomic DNA, PCR-mediated amplification of the 16S rDNA and direct sequencing of the purified PCR product were carried out according to Rainey et al. (1996). The sequence reaction mixtures were electrophoresed using a model 373A automatic DNA sequencer (Applied Biosystems). The partial 16S rDNA sequences were aligned with published sequences obtained from the EMBL Nucleotide Sequence Database (Cambridge, UK) and the Ribosomal Database Project (RDP) using the ae2 editor (Maidak et al., 1996) and similarity values determined.

2.3.7. Nucleotide sequence accession numbers.

The 16S rDNA sequences determined in this study were deposited in the EMBL database (Cambridge, United Kingdom) under the following accession numbers: ML1 - AJ 318407; ML10 - AJ 315686; ML14 - AJ 315689; ML19 - AJ 315688; ML20 - AJ

315694; ML21 - AJ 315695; ML30 - AJ 315684; ML35 - AJ 315693; ML36 - AJ 315692; ML45 - AJ 315685; ML3(repr.) - AJ 315691; ML42(repr.) - AJ 315683; $ML4^T$(repr.) - AJ 315687; ML22(repr.) - AJ 315690; ML37(repr.) - AJ 315697; $ML6^T$(repr) - AJ 315682.

2.4. Results and discussion.

2.4.1. Enumeration and isolation.

In a previous study it was found that the biomass of heterotrophic bacteria remained almost constant around 100 μg of C/l until July, however, during late summer and autumn it increased exponentially. This was caused by an increase in both bacterial cell numbers and bacterial cell volumes (Overman et al., 1996). Integrated bacterial production reached a transient peak in May and increased continuously and steeply between July and November, parallel to the increase in bacterial biomass (Overman et al., 1996). Therefore in October 1997, when our samples were collected, the heterotrophic (facultative and obligate) bacterial community was near its maximum. Dissolved oxygen (DO) profiles of Mahoney Lake showed that the lake is oxic to ~7 m depth, below which oxygen decreases rapidly to zero by 8 m (Northcote and Hall, 1983; Overman et al., 1991). On October 3, 1997, the chemocline occured at a depth of 8.35 m and extended down to 9.0 m, and the temperature and salinity were 18° C and 22 ppT at a depth of 8.5 m, respectively. On this date at 0 m (surface), temperature was 13.8° C, DO - 8.6 mg/l, salinity - 5 ppT; at 3 m, temperature was 25.3° C, DO - 12.8 mg/l, salinity - 20 ppT; and

at 5 m, temperature was 28° C, DO - 2.6 mg/l, and salinity - 22 ppT. Therefore the 3 and 5 m depth samples that we used were aerobic.

All APB require oxygen and organic carbon. Thus the reason why no selective medium has been developed to isolate APB is because many nonphototrophic microorganisms grow well on aerobic organic media. We designed media that should satisfy bacterial needs for most growth factors and be close to the natural parameters of Mahoney Lake at various depths. Rich media have been used previously for the enumeration of aerobic phototrophs (Yurkov and Beatty, 1998a). Because Mahoney Lake is mainly a sodium sulfate dominated lake with a mixolimnion SO_4^{2-} content ranging from 5 g/l at the surface to 14 g/l at 5 m depth (Hall and Northcote, 1986), medium N1, which has a high content of Na_2SO_4 (see section 2.3), was used to enumerate bacteria that require high amounts of sulfates. Medium N2 was devised to isolate and enumerate strains that prefer a NaCl-enriched medium for growth. Medium N4, which contains a very high concentration of Na_2SO_4 (50 g/l) was used to detect bacteria that either require or are resistant to such high sulfate concentrations.

Data were collected on the abundance of the following groups of microorganisms that were capable of forming colonies on the media employed: 1) aerobic noncolored bacteria; 2) aerobic pigmented bacteria; 3) APB; and 4) facultatively aerobic purple nonsulfur bacteria.

The results are summarized in Table 2.1 and 2.2. All samples analyzed gave rise to many non-pigmented colonies (representative of 2513 to 31500 cells/ml). The color of bacterial colonies indicative of the presence of carotenoids was used to identify

Table 2.1. Distribution and enumeration of pigmented cells isolated from Mahoney Lake.

Cell color	Media	Abundance (CFU/ml)			% of cells containing BChl		
		Surface	3m	5m	Surface	3m	5m
Non-colored	N1	7850	2513	9400	NA	NA	NA
	N2	3240	3483	3785	NA	NA	NA
	N4	8475	28800	31500	NA	NA	NA
Pink-purple	N1	0	120	4555	NA	60	0
	N2	0	475	1850	NA	36.4	0
	N4	790	2785	3000	28.6	11.1	58.4
Orange-red	N1	4233	0	3950	6.7	NA	0
	N2	1360	50	2000	50	0	0
	N4	480	70	650	0	0	25
Yellow	N1	1205	0	0	0	NA	NA
	N2	265	0	0	0	NA	NA
	N4	200	0	0	37.5	NA	NA
Brown-red	N1	2600	220	686	79.4	62.5	75
	N2	ND	0	200	NA	NA	0
	N4	240	1330	1600	100	53.3	100

ND, not determined; NA, not applicable.

Table 2.2. Some determinative characteristics of BChl *a* containing strains isolated from Mahoney Lake.

Strain	Isolated from	Color	*In vivo* carotenoid peaks (nm)	*In vivo* BChl peaks (nm)
ML14	Surface	Brown-orange	465	804, 870
ML15	Surface	Brown-orange	420, 464	802, 861
ML17	Surface	Pink-purple	408, 484	805, 866
ML19	Surface	Brown-orange	462, 488	807, 867
ML20	Surface	Yellow	427, 458, 488	803, 868
ML21	Surface	Brown-red	461, 488, 528	805, 866
ML22	Surface	Brown-red	464, 487	755, 805, 864
ML23	Surface	Brown-red	464, 488	806, 864
ML30	Surface	Pink	460	873
ML32[a]	Surface	Brown-red	468, 489	749, 806, 867
ML34	Surface	Brown-orange	464, 487	804, 869
ML35[b]	Surface	Yellow	430, 459, 488	805, 870
ML1	3m	Brown-red	462, 488	762, 809, 869
ML3	3m	Orange-brown	468	804, 860
ML4[T]	3m	Brown-red	466, 491	808, 866
ML6[Tc]	3m	Purple	408, 484	805, 870
ML10	3m	Pink	475, 505	866
ML37[d]	3m	Pink	480	859
ML29	5m	Pink	409	802, 861
ML42[e]	5m	Pink	451, 479, 510	803, 848, 880
ML45	5m	Greyish-yellow	409	806, 870

[a] represents group including ML31; [b] represents group including ML36; [c] represents group including ML16, ML18, ML33, ML38, ML39, ML40, ML44; [d] represents group including ML46, ML47; [e] represents group including ML43.

Table 2.2. Some determinative characteristics of BChl *a* containing strains isolated from Mahoney Lake (Continued).

Strain	Morphology
ML14	Short ovoid rods, chains of 2-3 cells.
ML15	Ovoid, thick rods, many long chains.
ML17	Ovoid rods.
ML19	Small, short ovoid or almost coccoid cells forming very long curved motile chains, connected by bubble-like structures. Produce prosthecae.
ML20	Thick ovoid rods, forming small chains.
ML21	Small, slightly curved rods, form short curved chains.
ML22	Extremely long thin rods, form chains. Cells often develop a small bubble like structure at one end.
ML23	Long rods, forming long straight chains.
ML30	Ovoid short rods, some chains of 2-3 cells.
ML32[a]	Ovoid short rods, some chains of 2-6 cells.
ML34	Ovoid short rods, some chains of 2-6 cells.
ML35[b]	Small ovoid rods, forming short chains.
ML1	Ovoid cells
ML3	Ovoid rods, forming chains. Production of buds and tendency for branching.
ML4[T]	Rods. Aggregates of cells in presumably polysaccharide matrix. Bead-like formations.
ML6[Tc]	Two types of cells, elongated rods, and bean shaped cells.
ML10	Large ovoid rods or elongated cells. Produce gas vacuoles.
ML37[d]	Long thin cells.
ML29	Pleomorphic
ML42[e]	Short ovoid cells, forms long chains.
ML45	Small short ovoid cells, forms short chains.

[a] represents group including ML31; [b] represents group including ML36; [c] represents group including ML16, ML18, ML33, ML38, ML39, ML40, ML44; [d] represents group including ML46, ML47; [e] represents group including ML43.

Table 2.2. Some determinative characteristics of BChl *a* containing strains isolated from Mahoney Lake (Continued).

Strain	Motility	Anaerobic phototrophic growth		Medium of isolation
		RO	Non-S	
ML14	-	-	-	N1
ML15	+	-	-	N1
ML17	+	-	-	N1
ML19	+	-	-	N1
ML20	+	-	-	N1
ML21	+	-	-	N1
ML22	+	-	-	N1
ML23	-	-	-	N1
ML30	+	-	-	N4
ML 32[a]	+	-	-	N4
ML34	+	-	-	N4
ML35[b]	+	-	-	N4
ML1	+	-	-	N1
ML3	-	-	-	N1
ML4[T]	-	-	-	N1
ML6[Tc]	+	-	-	N1
ML10	+	-	-	N2
ML37[d]	+	-	-	N4
ML29	+	-	-	N2
ML42[e]	+	+	+	N4
ML45	+	-	-	N4

+, positive; -, negative; RO, rich organic media; Non-S, media for purple non-sulfur bacteria.

[a] represents group including ML31; [b] represents group including ML36; [c] represents group including ML16, ML18, ML33, ML38, ML39, ML40, ML44; [d] represents group including ML46, ML47; [e] represents group including ML43.

presumptive phototrophic bacteria, which were subsequently screened for the presence of BChl in absorption spectra. Most of the pigmented cells contained carotenoids but not BChl. BChl *a* was detected in 31% of pigmented strains isolated from surface samples, in 28% of pigmented strains from 3 m and in 22% of pigmented strains recovered from 5 m depth. These data are similar to those reported for freshwater and seawater microbial mats, and for pelagic marine environments including deep ocean hydrothermal vent samples where BChl-containing strains comprised 10-30% of pigmented bacteria (Yurkov and Gorlenko, 1990; Shiba et al., 1991; Yurkov and Van Gemerden, 1993; Yurkov and Beatty, 1998b). However the presence of BChl in different Mahoney Lake pigmented isolates did not correlate with depth of sample, colony color or media used (Table 2.1). Thus Bchl-containing species are dispersed throughout the mixolimnion.

2.4.2. Morphology and cytology.

Thirty seven strains that produce BChl *a* and carotenoids were isolated. Based on color, colony and cell morphology, cytology, absorption spectra and major physiological aspects, these 33 isolates were grouped as shown in Table 2.2. All purified strains form regular circular colonies on the surface of agar media, varying in size from 1-2 mm to 6-7 mm. Some isolates were found to be non-motile (Table 2.2), although most of the isolates are motile having one long (Fig. 2.1A, strain ML42 as example) or several wavy flagella (Fig. 2.2A, strain ML10 as example). All isolates stained Gram-negative and this cell wall organization was confirmed by electron microscopy (Figs. 2.1B; 2.2B, C; 2.3D,

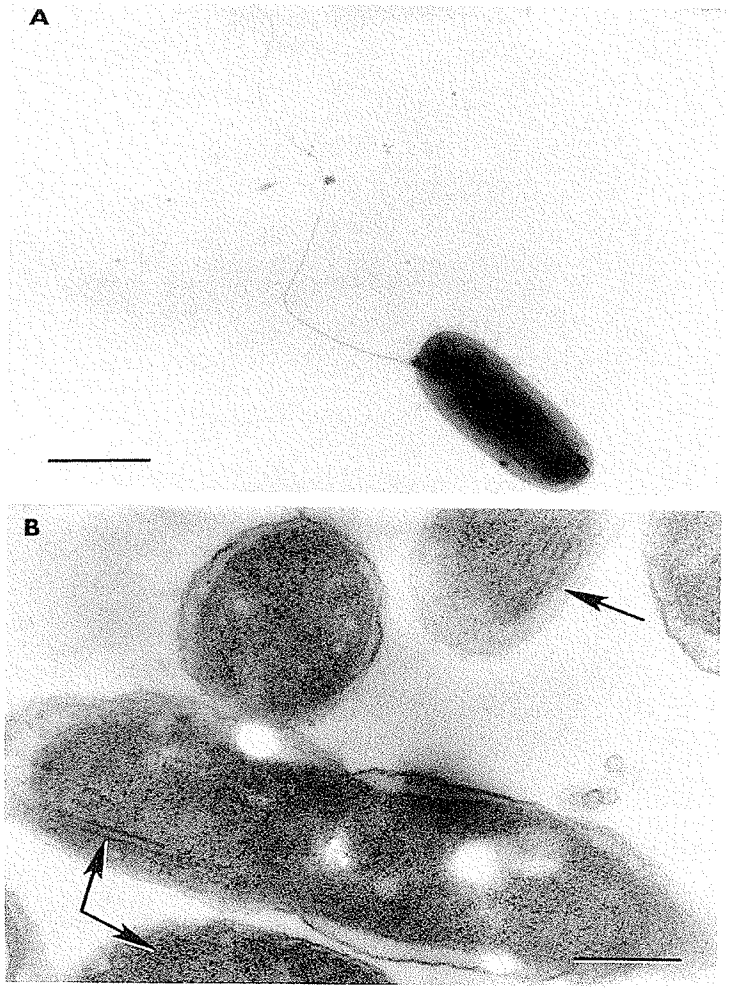

Fig. 2.1. Strain ML42. (A) Rod cell with one long flagellum. Negative staining. (B) Ultra-thin section of cells showing gram-negative cell wall and poorly developed photosynthetic intracytoplasmic membranes of thylakoid type (indicated by arrows). Bars: 0.5 μm (A), 0.25 μm (B).

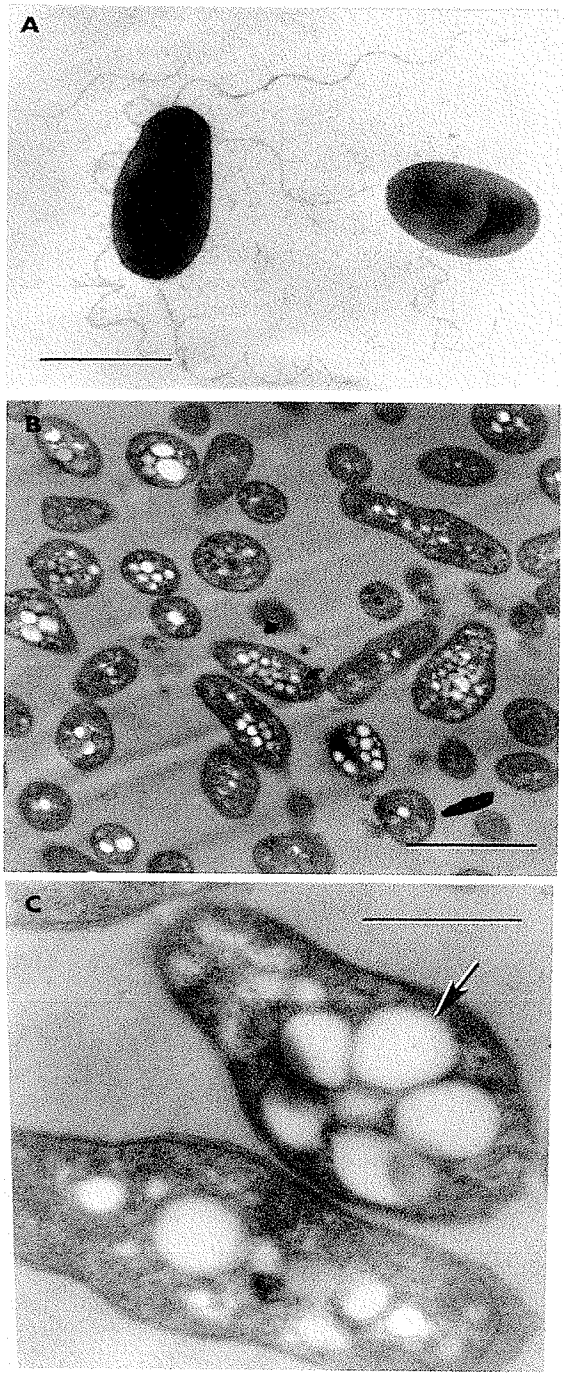

Fig. 2.2. Strain ML10. (A) Negatively stained cells with possible multiple wavy flagellation. (B, C) Ultra-thin sections of cells with extensive gas vacuole production (indicated by arrow). Bars: 1 μm (A), 2 μm (B), 0.5 μm (C).

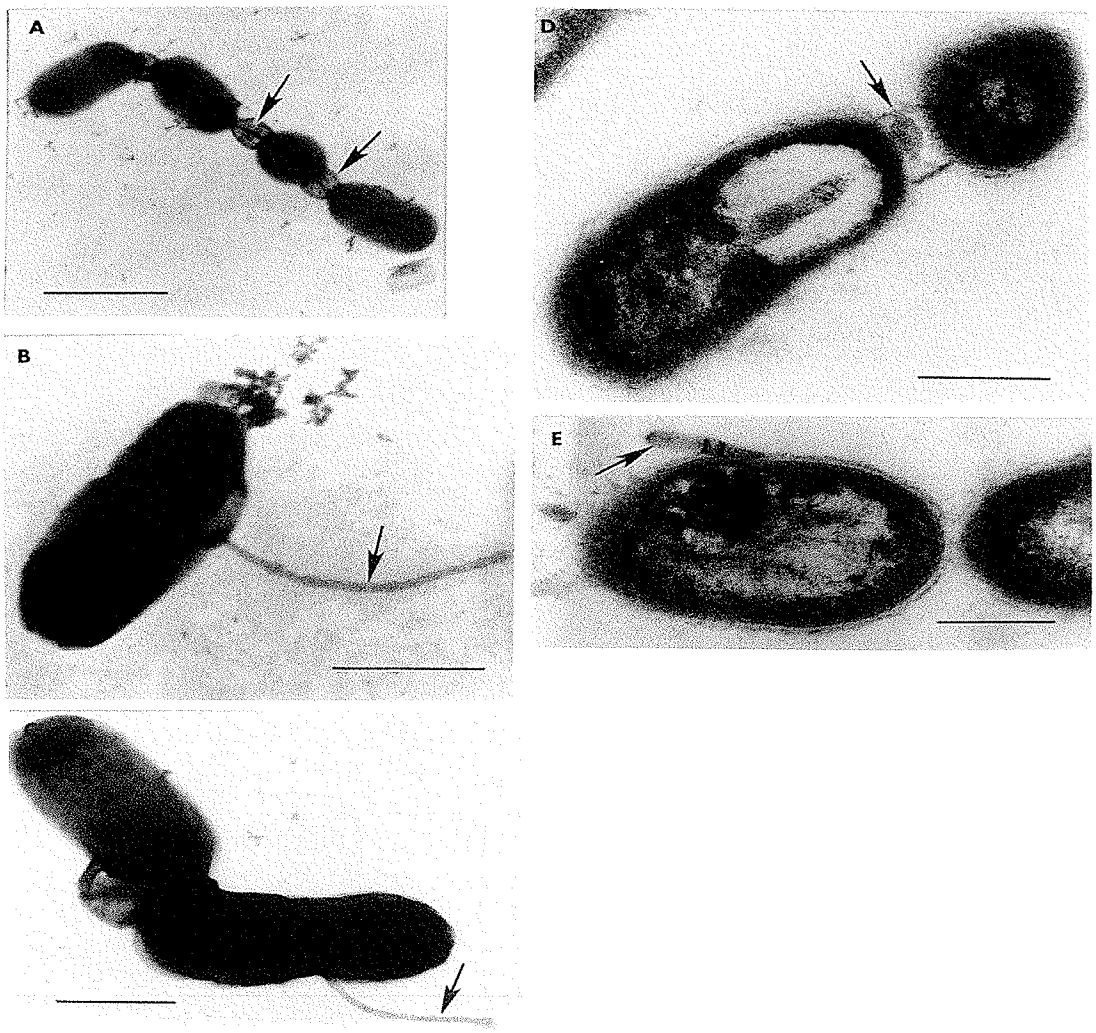

Fig. 2.3. Strain ML19. (A, D) Unusual form of cell connection. Bubble-like structures with micro-tubular "bridges" in the middle are indicated by arrows. (B, C) Prostheca formation is indicated by arrows. (E) Initiation of prostheca formation. (A, B and C) negatively stained cells and (D, E) ultra-thin sections of cells. Bars: 1 μm (A), 0.5 μm (B, C, E), 0.25 μm (D).

E; 4C and 5C, D; strains ML42, ML10, ML19, ML4^T and ML6^T, respectively as examples). Most of the strains reproduce by binary division. Strains ML1, ML3, ML6^T, ML20, ML21, ML22, ML23, ML35 and ML40 divide by both symmetric and asymmetric constrictions. Production of buds and a tendency for branching was found in strains ML3 and ML4^T.

Electron microscopy of thin sections of cells revealed that some strains accumulate storage materials. Strains ML1, ML3, ML4^T, ML6^T, ML10 and ML42 accumulated electron-clear granules attributed to polyhydroxyalkanoate compounds. Strains ML1 and ML3 accumulate electron-dense granules thought to be polyphosphate, whereas strain ML4^T produces capsule material, presumed to be composed of polysaccharides, that forms a matrix in which cells are embedded (Fig. 2.4A, strain ML4^T). Accumulation of storage materials such as polysaccharides, polyphosphates and polyhydroxyalkanoates by APB is quite common and has been discussed in a recent review (Yurkov and Beatty, 1998a).

A plethora of different cell shapes was observed among the new isolates under phase contrast light and electron microscopy (Table 2.2). The most interesting or unusual morphologies are presented in Figs 2.1, 2.2, 2.3, 2.4, 2.5 and 2.6.

The pink colored strain ML10 is motile and forms ovoid rods or elongated cells of irregular shape (Fig. 2.2A, B and C). Elongated cells are often organized in rosettes of 3-6 cells (not shown). Massive formation of presumably polyhydroxyalkanoate granules is a specific feature of this strain (Fig. 2.2B and C).

The brown-orange strain ML19 has ovoid or almost coccoid cells forming long curved motile chains (Fig. 2.3A and D). From electron micrographs it can be seen that

cells in chains are connected by some unknown bubble-like structures which have tubular connections in the middle. This type of connection has not been shown for previously described aerobic phototrophic species and deserves special study. Perhaps cells of strain ML19 exchange metabolites via these unusual structures. Another peculiarity of ML19 is the formation of prosthecae (Fig. 2.3B, C, and E). Prosthecae increase significantly the surface-to-volume ratio of a cell, which may confer an increased ability to take up nutrients and expel wastes, and may function to provide buoyancy (Madigan et al., 1997).

Ovoid rods of the brown-red strain ML4^T excrete large amounts of capsular material, which holds cells together in small aggregates. Cells also produce very unusual bead-like formations (Fig. 2.4A, B and C). Very often these "beads" are branched (Fig. 2.4A), and the size and shape of "beads" is variable. The functional role of these "beads" as well as possible life cycle changes in ML4^T requires further investigation.

A group of pink-purple strains, ML6^T, ML16, ML18, ML23, ML38, ML39, ML40 and ML44, shows very interesting morphology. In liquid and especially on solid media two types of cells are produced, elongated rods and bean shaped (vibrioid) cells (Fig. 2.5A, B, C and D), and both types of cells have pointed ends. Sometimes cells form a helical shape. This morphology is similar to that of the purple nonsulfur bacterium *Rhodocyclus purpureus* (Pfennig, 1978). Electron microscopy of negatively stained cells revealed electron-dense areas at their poles (Fig. 2.5A and B), and thin sections indicated polar zones of significantly enlarged periplasmic space in both vibrioid cells and rods. The significance of this enlarged periplasm at the cell ends is unclear.

Strain ML22 has extremely long, thin cells (Fig. 2.6A and B), and many cells develop a small bubble-like formation at one end. Although this structure has not been

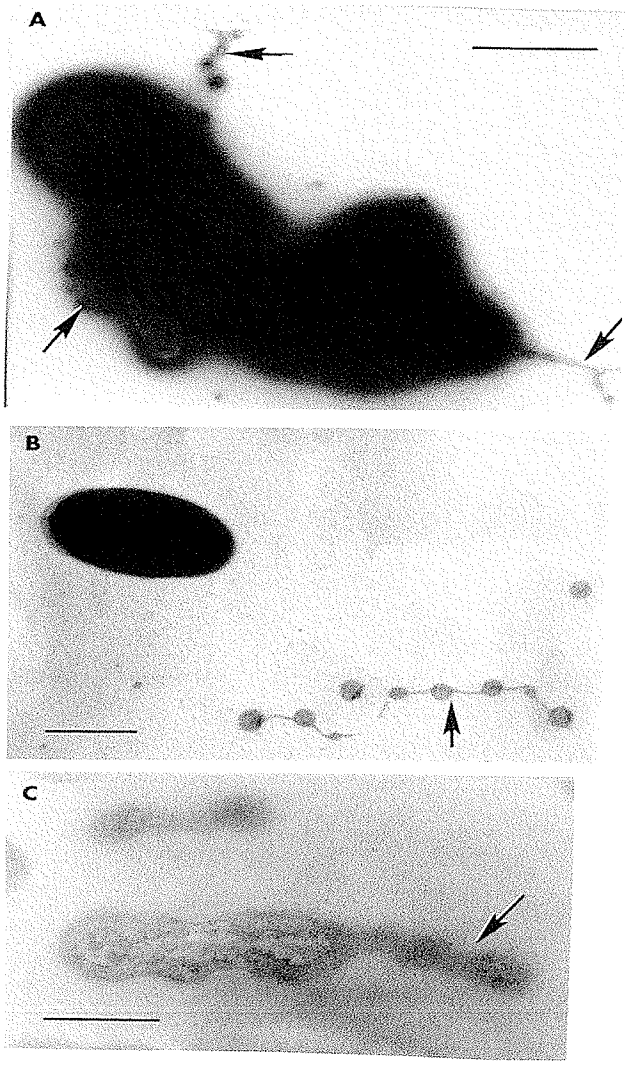

Fig. 2.4. Strain ML4^{T}. (A) Rod cells incorporated into presumably polysaccharide matrix, and chains of bead-like formations (indicated by arrows). (B) Ovoid cell and a chain of large "beads". (C) Elongated cell of irregular shape with prostheca-like formation (indicated by arrow). (A, B) negative staining and (C) ultra-thin section. Bars: 1μm (A), 0.25 μm (B), 0.5 μm (C).

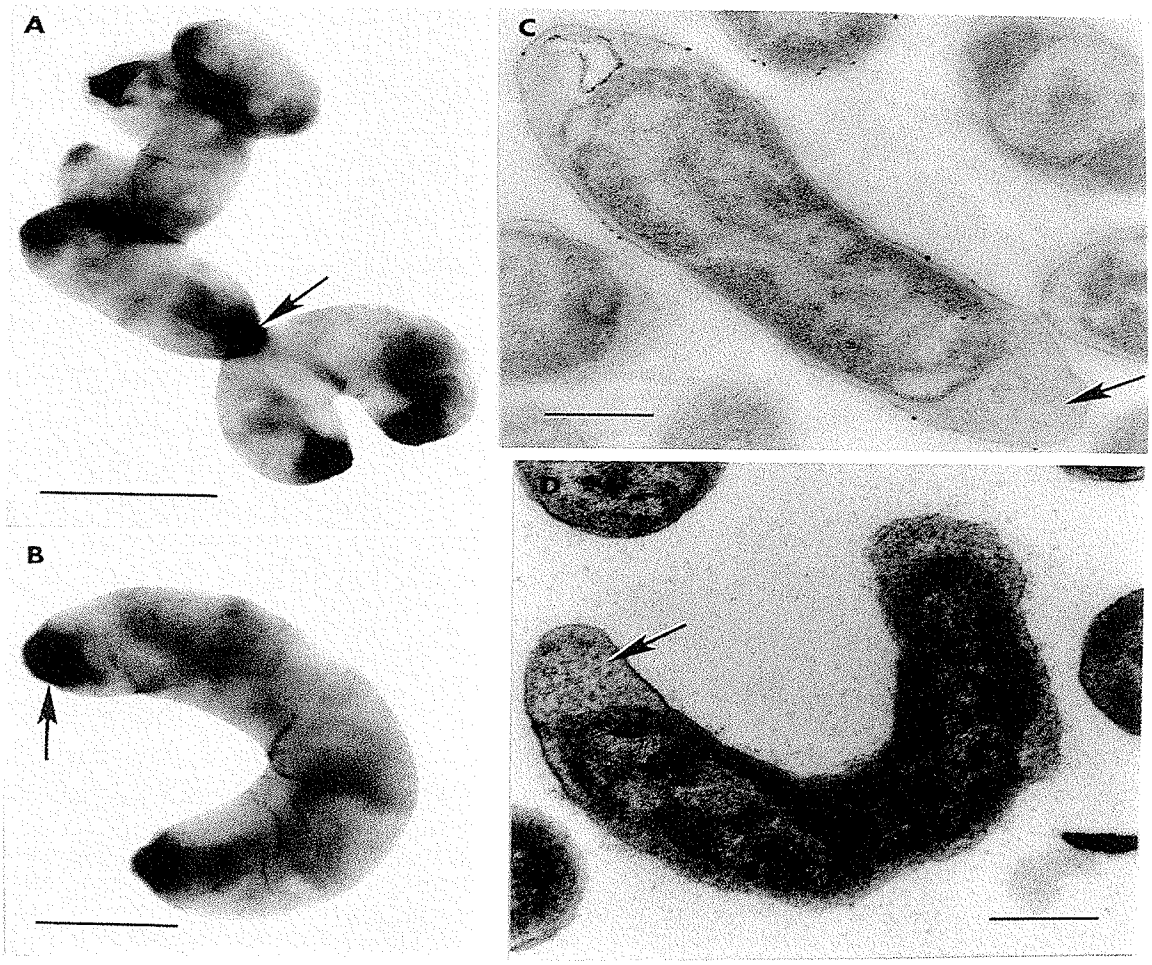

Fig. 2.5. Strain ML6^{T}. (A,B) Negative stained bean shaped and elongated cells with polar dark coloration of pointed ends. (C, D) Ultra-thin sections of cells show polar periplasmic modifications (confirming polar staining in A and B; indicated by arrows). Bars: 1 μm (A), 0.5 μm (B), 0.25 μm (C, D).

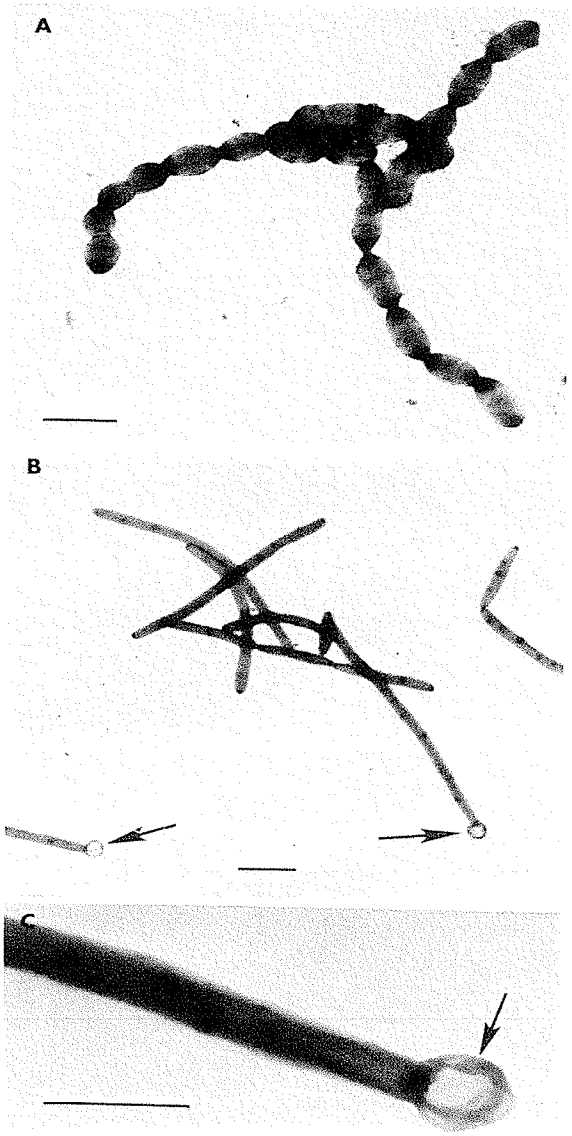

Fig. 2.6. Strain ML3 (A) which forms long chains of cells. (B, C) Long cells of strain ML22 producing small bubble-like structures of unknown function at end of cell (indicated by arrows). Bars: 2 μm (A, B), 1μm (C).

investigated further, one of the possible functions could be similar to the role of gas vacuoles in regulating cell buoyancy.

2.4.3. Photosynthetic apparatus.

The capability for anaerobic photosynthetic growth (with tungsten filament lamp illumination level of about 30 microeinsteins/m^2/s) of the BChl-containing isolates was tested in completely filled screw-cap test tubes and in agar (1%) deeps by using media (containing H_2S, or $Na_2S_2O_3$, and CO_2 with or without acetate) for purple sulfur bacteria, or media (containing acetate, malate or succinate as sole carbon source) for nonsulfur bacteria. Anaerobic photosynthetic growth was also tested in the original liquid medium (N1, N2 or N4) used for isolation (see Table 2.2). Almost all of the isolates did not grow anaerobically in the light in these media, which leads us to designate them as obligate APB (Yurkov and Beatty, 1998a).

The isolates ML42 and ML43 grew photosynthetically (anaerobically) in purple nonsulfur bacterial media, whereas they did not grow in the purple sulfur bacterial medium that contained sodium sulfide (0.3 g/l). This suggests that this concentration of sulfide is toxic for ML42 and ML43, and so we conclude that these two isolates are members of the purple non-sulfur bacteria. Anaerobic photosynthetic growth (although slow) yielded the highest cell densities in the liquid medium N4 (see section 2.3), whereas weaker growth occurred in the medium designed for purple non-sulfur bacteria. In all media, aerobically grown cultures were pink, whereas cultures grown anaerobically with illumination were light-brown, indicating a change in pigment composition. Such color difference between aerobically and anaerobically grown cultures is common for

photosynthetic bacteria, and is due to a change in carotenoid composition and enhanced production of BChl (Imhoff and Truper, 1992). Transfer of cells from aerobic plates to anaerobic photosynthetic conditions resulted in long lag phases of at least 48 h, whereas cells previously cultured under anaerobic photosynthetic conditions transferred to similar anaerobic photosynthetic conditions did not show this long lag phase.

Cells of ML42 and ML43 grown photosynthetically (anaerobically) contain small amounts of intracytoplasmic membranes (ICM) of thylakoid type, which we assume contain the photosynthetic apparatus (Fig. 2.1B). The absorption spectra of strains ML42 and ML43 grown under different oxygen conditions and media were significantly different (Fig. 2.7), although still indicative of RC and LH complexes. As can be seen from the *in vivo* absorption spectra, the LH apparatus consists of two types of antenna: an absorption peak at 880 nm that we attribute to an unusually red-shifted LH complex I; and absorption peaks at 804 and 848 nm that we assign to a peripheral LH complex II (Fig. 2.7A, B and C). The long wavelength absorption peak of LH I indicates an unusual protein environment of BChl in this complex.

The relative amounts of the LH complexes in ML42 and ML43 seem to depend on the oxygen concentration and medium composition. This conclusion is reached because in aerobically grown cells the LH I (880 nm) peak amplitude was much greater than that of the LH II (804 and 848 nm) peaks, such that the LH II 848 nm peak was a shoulder on the LH I 880 nm peak (Fig. 2.7B). In contrast, under anaerobic photosynthetic conditions in medium N4 cells produced both complexes at about similar levels, as indicated by the amplitudes of the LH II 848 nm and the LH I 880 nm peaks

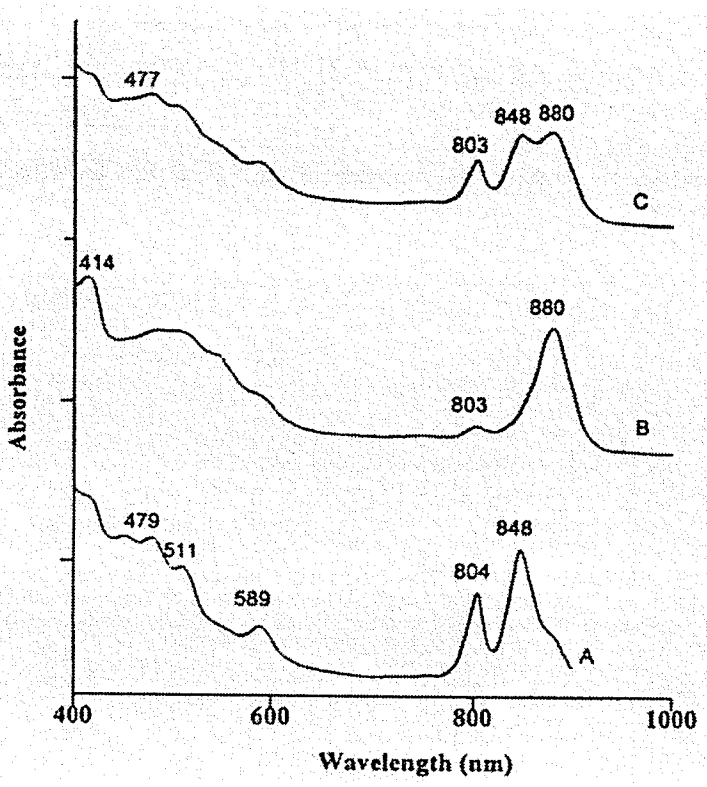

Fig. 2.7. Absorption spectra of the photosynthetic apparatus of strain ML42 synthesized under different growth conditions. Cells were grown in: (A) Liquid medium for purple non-sulfur bacteria, anaerobically; (B) Medium N4, aerobically on agar surface; (C) Liquid medium N4, anaerobically.

(Fig. 7C). After anaerobic, photosynthetic growth to the stationary phase in the medium for purple nonsulfur bacteria the LH II peaks (at 804 nm and 848 nm) predominated, with the LH I absorption peak at 880 nm present as a small shoulder (Fig. 2.7A). Although the influence of oxygen and light intensities on LH complexes in purple photosynthetic bacteria is well known, we do not know whether the ML42 and ML43 isolates produce different forms of LH I and LH II complexes dependent on salinity, medium composition, temperature or light intensity, as reported for *Rhodopseudomonas acidophila* and *R. palustris* (Bissig et al., 1990; Evans et al., 1990; Gardiner et al., 1992; Tadros et al., 1993). The carotenoid composition (absorption peaks between 400 and 550 nm) as well as the amount of a presumed cyt (peak at 414 nm) also depended on the oxygen availability (Fig. 2.7 A, B and C).

The absorption characteristics of obligately aerobic phototrophic strains are summarized in Table 2.2. The differences in carotenoid composition (absorption peaks in blue and green regions of the light spectrum between 400 and 550 nm) and BChl-protein complexes (absorption peaks in the infrared region of the light spectrum between 800 and 880 nm) are consistent with the colorful variety of isolates (from yellow to brown-red). We have detected at least eight variants of the LH complexes among these isolates (Fig. 2.8). The most interesting is the LH complex found in the eight strains that produce vibrioid cells (see section 2.4.2). These strains seem to have an unusual LH II complex that has one absorption peak at ~805 nm (Fig. 2.8G, strain ML6^T as example). This absorption profile has been found only in the aerobic phototrophic genera *Roseobacter* and *Rubrimonas* (Shiba, 1991; Nishimura et al., 1994; Suzuki et al., 1999). However,

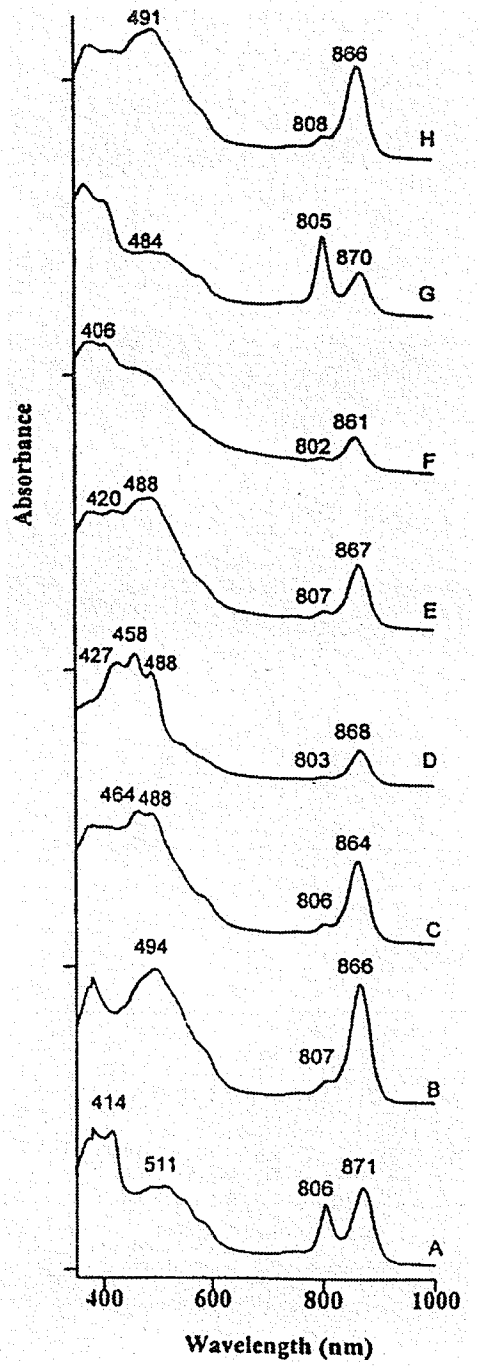

Fig. 2.8. Diversity of photosynthetic light harvesting complexes and carotenoid compositions found in new strains as shown by absorption spectra: (A) ML40; (B) ML31; (C) ML23; (D) ML20; (E) ML19; (F) ML15; (G) $ML6^T$ and (H) $ML4^T$.

Roseobacter and *Rubrimonas* species have morphological, cytological and major physiological properties different from our isolates.

2.4.4. Nutritional and other properties.

A variety of diagnostic growth and physiological properties of the Mahoney Lake isolates are presented in Table 2.3.

All strains were catalase and oxidase positive. Many strains hydrolyzed gelatin, Tween 60 and starch indicating the presence of gelatinase, lipolytic and amylolytic activities (Table 2.3). Only three strains, ML10, ML45 and ML 46, did not hydrolyze gelatin, Tween 60 or starch. Isolates from Mahoney Lake are able to grow over a wide range of temperatures, with all strains exhibiting growth at 5° C, and optimum growth occuring at 28° C. All strains, except ML16, ML17, ML18, and ML21 are able to grow at 37°C, and strain ML10 is capable of growth as high as 45°C.

The pH of the Mahoney Lake mixolimnion shows a vertical variation from around pH 9 at the surface to about pH 8 near the chemocline, and all of the isolates tolerated a wide range of pH for growth. Most strains grew optimally without any difference in the range of pH 5.5 to 10. Strains ML6^T, ML16, ML17, ML18, ML30, ML33, ML38, ML39, ML40 and ML44 preferred a slightly higher pH with a minimum value that allowed growth of pH 6. All of these isolates were able to grow at pH as high as 11, and some of them grew equally well at pH 11 as at lower pH. The three strains ML34, ML35 and ML36 tolerated the broadest range of pH from pH 5 to pH 11.

Table 2.3. Comparative physiological characteristics of the aerobic phototrophic strains isolated from Mahoney Lake.

Characteristic	Strain								
	ML1	ML3	ML4^T	ML6^T	ML10	ML14	ML15	ML16	ML17
Growth at pH									
5.0	-	-	-	-	-	-	-	-	-
5.5	++	++	++	-	-	W	+	-	-
6.0	++	++	++	++	++	W	++	++	++
7.0	++	++	++	++	++	+	++	++	++
8.0	++	++	++	++	++	+	++	++	++
9.0	++	++	++	++	++	+	++	++	++
9.5	++	++	++	++	++	++	++	++	++
10.0	+	+	++	++	++	++	++	++	++
11.0	-	-	-	++	-	-	-	++	++
Utilization of									
Acetate	++	W	++	+	+	+	-	+	+
Pyruvate	+	+	++	+	W	+	++	+	-
Glutamate	++	++	++	+	W	++	+	+	-
Butyrate	++	++	++	-	-	+	W	+	-
Citrate	-	-	-	+	-	-	-	-	-
Malate	-	-	++	+	W	-	-	+	-
Succinate	-	+	++	+	W	-	-	+	-
Lactate	+	-	+	+	W	-	-	+	-
Formate	-	-	-	-	-	+	-	-	-
Fructose	-	-	-	++	-	-	-	++	+
Glucose	-	-	++	++	+	+	-	++	+
Ethanol	-	-	-	-	-	-	-	-	-
Methanol	-	-	-	-	-	-	-	-	-
Yeast extract	++	++	++	++	++	+	++	++	++
Hydrolysis of									
Starch	-	W	W	+	-	+	+	+	NG
Gelatin	-	-	-	+	-	+	+	+	+
Tween 60	++	++	++	-	-	++	++	-	-
Vitamin requirement									
Thiamine	W	-	-	ND	ND	-	-	ND	ND
Biotin	-	+	+	ND	ND	+	+	ND	ND
Nicotinic acid	W	-	-	ND	ND	-	-	ND	ND
Vitamin B12	-	+	+	ND	ND	+	+	ND	ND
Antibiotic Sensitivity									
Chloramphenicol	+	+	+	+	+	+	+	+	+
Penicillin G	+	+	-	-	+	-	+	-	-
Streptomycin	-	-	-	+	+	-	-	+	+
Polymixin B	-	-	-	+	-	-	-	+	+
Tetracycline	+	+	+	+	+	+	+	+	+
Ampicillin	-	-	-	-	+	-	-	-	-
Kanamycin	+	+	+	+	+	+	+	+	+
Nalidixic acid	-	-	-	-	-	-	-	-	-

+, substrate is utilized, substrate is hydrolysed, vitamin required or antibiotic sensitive; ++, substrate is utilized for very good growth; -, substrate is not utilized, substrate is not hydrolysed, vitamin is not required or antibiotic resistance; W, very weak growth; ND, not determined; NG, no growth.

Table 2.3. Comparative physiological characteristics of the aerobic phototrophic strains isolated from Mahoney Lake (Continued).

Characteristic	Strain								
	ML18	ML19	ML20	ML21	ML22	ML23	ML29	ML30	ML31
Growth at pH									
5.0	-	-	W	-	-	-	-	-	-
5.5	-	++	++	-	++	++	-	-	W
6.0	++	++	++	++	++	++	-	++	++
7.0	++	++	++	++	++	++	++	++	++
8.0	++	++	++	++	++	++	++	++	++
9.0	++	++	++	++	++	++	++	++	++
9.5	++	++	++	++	++	++	++	++	++
10.0	++	++	++	++	++	++	++	++	+
11.0	++	-	-	-	-	W	+	+	-
Utilization of									
Acetate	+	+	+	+	W	W	+	+	++
Pyruvate	+	++	W	W	+	++	+	-	++
Glutamate	+	++	++	++	+	++	+	-	++
Butyrate	-	++	++	++	+	-	-	-	++
Citrate	+	-	-	-	-	-	+	-	+
Malate	+	+	-	-	-	-	W	-	++
Succinate	+	+	-	W	-	-	-	-	++
Lactate	+	W	-	-	-	-	+	-	+
Formate	-	-	-	-	-	-	-	-	+
Fructose	++	-	-	-	-	-	+	-	++
Glucose	++	-	+	+	+	++	+	-	++
Ethanol	-	-	-	-	-	-	-	-	W
Methanol	-	-	-	-	-	-	-	-	W
Yeast extract	++	++	++	+	++	++	++	++	++
Hydrolysis of									
Starch	NG	+	-	+	+	W	+	-	+
Gelatin	+	+	+	NG	-	-	+	-	-
Tween 60	-	++	++	++	++	++	+	+	++
Vitamin requirement									
Thiamine	ND	-	-	-	-	-	ND	ND	-
Biotin	ND	+	W	+	+	+	ND	ND	W
Nicotinic acid	ND	-	-	-	-	-	ND	ND	-
Vitamin B12	ND	+	+	+	+	+	ND	ND	+
Antibiotic Sensitivity									
Chloramphenicol	+	+	+	+	+	+	+	+	+
Penicillin G	-	-	-	+	+	+	-	+	+
Streptomycin	-	-	-	-	-	-	+	+	-
Polymixin B	+	-	-	-	-	-	+	-	-
Tetracycline	+	+	+	+	+	+	+	+	+
Ampicillin	-	-	-	+	-	-	-	-	+
Kanamycin	-	+	+	+	+	+	+	-	+
Nalidixic acid	-	-	-	-	-	-	-	-	-

+, substrate is utilized, substrate is hydrolysed, vitamin required or antibiotic sensitive; ++, substrate is utilized for very good growth; -, substrate is not utilized, substrate is not hydrolysed, vitamin is not required or antibiotic resistance; W, very weak growth; ND, not determined; NG, no growth.

Table 2.3. Comparative physiological characteristics of the aerobic phototrophic strains isolated from Mahoney Lake (Continued).

Characteristic	Strain								
	ML32	ML33	ML34	ML35	ML36	ML37	ML38	ML39	ML40
Growth at pH									
5.0	-	-	+	+	+	-	-	-	-
5.5	++	-	++	+	++	-	-	+	-
6.0	++	++	++	++	++	-	++	++	++
7.0	++	++	++	++	++	++	++	++	++
8.0	++	++	++	++	++	++	++	++	++
9.0	++	++	++	++	++	++	++	++	++
9.5	++	++	++	++	+	++	++	++	++
10.0	+	++	++	++	+	++	++	++	++
11.0	-	+	+	+	+	+	+	+	+
Utilization of									
Acetate	++	-	W	+	-	-	-	-	W
Pyruvate	++	-	W	-	-	-	+	-	W
Glutamate	++	-	++	-	++	-	+	-	-
Butyrate	++	-	++	-	+	-	-	-	-
Citrate	+	W	-	-	-	-	+	+	+
Malate	+	-	-	-	-	-	+	-	-
Succinate	+	-	W	-	-	-	+	-	-
Lactate	+	-	-	-	-	-	+	-	W
Formate	+	-	-	-	-	-	-	-	-
Fructose	+	-	-	-	-	-	+	+	+
Glucose	++	-	++	-	-	-	+	+	-
Ethanol	W	-	-	-	-	-	-	-	-
Methanol	W	-	-	-	-	-	-	-	-
Yeast extract	++	+	++	++	++	++	++	++	++
Hydrolysis of									
Starch	-	-	-	-	-	-	-	-	-
Gelatin	+	+	-	+	+	+	+	+	+
Tween 60	++	-	++	++	++	+	+	NG	+
Vitamin requirement									
Thiamine	-	ND	-	-	-	-	ND	ND	ND
Biotin	W	ND	+	+	W	+	ND	ND	ND
Nicotinic acid	-	ND	-	-	-	-	ND	ND	ND
Vitamin B12	+	ND	+	+	+	W	ND	ND	ND
Antibiotic Sensitivity									
Chloramphenicol	+	+	+	+	+	+	+	+	+
Penicillin G	+	-	+	-	+	-	-	-	-
Streptomycin	-	-	-	-	-	-	-	-	-
Polymixin B	-	+	-	-	-	-	+	+	+
Tetracycline	+	+	+	+	+	+	+	+	+
Ampicillin	-	-	-	-	-	+	-	-	-
Kanamycin	+	-	-	-	+	-	-	-	-
Nalidixic acid	-	-	-	-	-	+	-	-	-

+, substrate is utilized, substrate is hydrolysed, vitamin required or antibiotic sensitive; ++, substrate is utilized for very good growth; -, substrate is not utilized, substrate is not hydrolysed, vitamin is not required or antibiotic resistance; W, very weak growth; ND, not determined; no growth.

Table 2.3. Comparative physiological characteristics of the aerobic phototrophic strains isolated from Mahoney Lake (Continued).

Characteristic	Strain					
	ML42	ML43	ML44	ML45	ML46	ML47
Growth at pH						
5.0	-	-	-	-	-	-
5.5	-	-	-	-	-	-
6.0	++	++	++	W	-	-
7.0	++	++	++	++	++	++
8.0	++	++	++	++	++	++
9.0	++	++	++	++	++	++
9.5	++	++	++	++	++	++
10.0	++	++	++	++	++	++
11.0	-	-	+	-	++	++
Utilization of						
Acetate	-	-	-	+	-	-
Pyruvate	-	+	W	++	-	-
Glutamate	+	+	W	+	+	-
Butyrate	-	-	-	-	-	-
Citrate	-	-	+	-	-	-
Malate	-	-	-	-	-	-
Succinate	-	-	W	-	-	-
Lactate	-	+	+	+	-	-
Formate	-	-	-	-	-	-
Fructose	-	-	+	-	-	-
Glucose	+	W	+	-	-	-
Ethanol	-	-	-	-	-	-
Methanol	-	-	-	-	-	-
Yeast extract	++	++	++	++	+	++
Hydrolysis of						
Starch	W	W	-	-	-	-
Gelatin	-	-	+	-	-	+
Tween 60	+	+	+	-	-	+
Vitamin requirement						
Thiamine	ND	ND	ND	+	-	-
Biotin	ND	ND	ND	+	+	+
Nicotinic acid	ND	ND	ND	+	-	-
Vitamin B12	ND	ND	ND	-	W	W
Antibiotic Sensitivity						
Chloramphenicol	+	+	+	+	+	+
Penicillin G	-	-	-	-	-	-
Streptomycin	-	+	-	+	-	-
Polymixin B	+	+	+	+	-	-
Tetracycline	-	+	-	+	+	+
Ampicillin	-	-	-	-	-	+
Kanamycin	-	-	-	-	-	-
Nalidixic acid	-	-	-	-	+	+

+, substrate is utilized, substrate is hydrolysed, vitamin required or antibiotic sensitive; ++, substrate is utilized for very good growth; -, substrate is not utilized, substrate is not hydrolysed, vitamin is not required or antibiotic resistance; W, very weak growth; ND, not determined.

The isolates were screened for their ability to utilize various organic compounds as sole carbon sources. In general, they can be divided into three groups. The first and largest group of strains (ML1, ML3, ML4^T, ML6^T, ML10, ML14, ML16, ML18, ML19, ML20, ML21, ML22, ML23, ML29, ML31, Ml32, ML34, ML38, ML40, ML43, ML44 and ML45) utilize a wide variety of organic compounds as sole carbon source. This is typical for aerobic phototrophs associated with environments rich in organic matter (Yurkov and Beatty, 1998a). The second group contains strains ML15, ML17, ML30, ML35, ML36, ML39, ML42 and ML46 that have very restricted metabolic abilities. These isolates utilize a limited number of organic compounds as a sole carbon source with best growth occurring when yeast extract, casamino acids or bactopeptone were provided. The third and smallest group of strains (ML33, ML37 and ML47) did not grow in media that contained only single organic compounds (organic acids or sugars), whereas growth was obtained in media supplemented with yeast extract or casamino acids.

Surprisingly, many of the aerobic phototrophic isolates demonstrated a requirement for biotin and/or vitamin B_{12}. This requirement is unusual for the APB, as only one genus *Roseobacter* has been shown to be vitamin dependent (Shiba, 1991), although the growth of *Citromicrobium bathyomarinum* is stimulated by the addition of biotin to a minimal medium (Yurkov et al., 1999).

As is common for the APB, there was variable resistance and sensitivity to several antibiotics (Table 2.3).

2.4.5. Effect of salinity.

Because Mahoney Lake shows considerable vertical variation in salinity (4-40‰) (Hall and Northcote, 1986), salinity could be an important environmental parameter that influences the growth and survival of microorganisms in this econiche. For this reason it was of interest to study the influence of salinity on the growth of the newly isolated strains of aerobic phototrophs. The salt requirement and tolerance of strains isolated from different depths were tested in aerated liquid media (N1, N2, N3 and N4; see section 2.3) containing concentrations of NaCl or Na_2SO_4 from 0.5% to 15% (Table 2.4).

The Mahoney Lake isolates exhibited a wide salt tolerance. Our experiments revealed that many strains grew in media with no NaCl or Na_2SO_4 added, however none of the investigated strains were obligately freshwater (Table 2.4; Fig. 2.9). Only one strain (ML3) appeared well suited to growth in the absence of added salts, showing little difference in growth as compared to saline media (Fig. 2.9B). Most of the strains able to grow in the absence of a salt supplement showed much weaker growth than on the same medium supplemented with a salt. Several strains ($ML6^T$, ML16, ML18, ML29, ML33, ML37, ML38, ML39, ML40, ML42, ML43, ML44, ML45 and ML46) could be described as obligately halophilic, because they did not grow in non-supplemented media and very slow growth occurred at NaCl or Na_2SO_4 concentrations not lower than 0.5% (Table 2.4, Fig 2.9A). Strains ML15, ML19, ML21, ML22 and ML23 grew in all media tested with slight preference for additional sulfate, whereas strains $ML4^T$, ML32, ML35, ML38, ML42, and ML45 grew best in the highly halophilic (supplemented with Na_2SO_4) medium N4 (Fig. 2.9C).

Table 2.4. Salt requirement and tolerance of strains isolated from different depths.

Strain	Depth	% Salt
		0 2 4 6 8 10 12 14
ML17	S	
ML22	S	
ML23	S	
ML19	S	
ML20	S	
ML21	S	
ML31	S	
ML32	S	
ML35	S	
ML36	S	
ML30	S	
ML1	3m	
ML3	3m	
ML4	3m	
ML10	3m	
ML42	5m	
ML43	5m	
ML45	5m	
ML29	5m	
ML16	S	
ML18	S	
ML33	S	
ML38	3m	
ML39	3m	
ML40	3m	
ML44	5m	
ML37	3m	
ML46	5m	
ML 6	3m	

S, surface; ····· NaCl; — Na_2SO_4

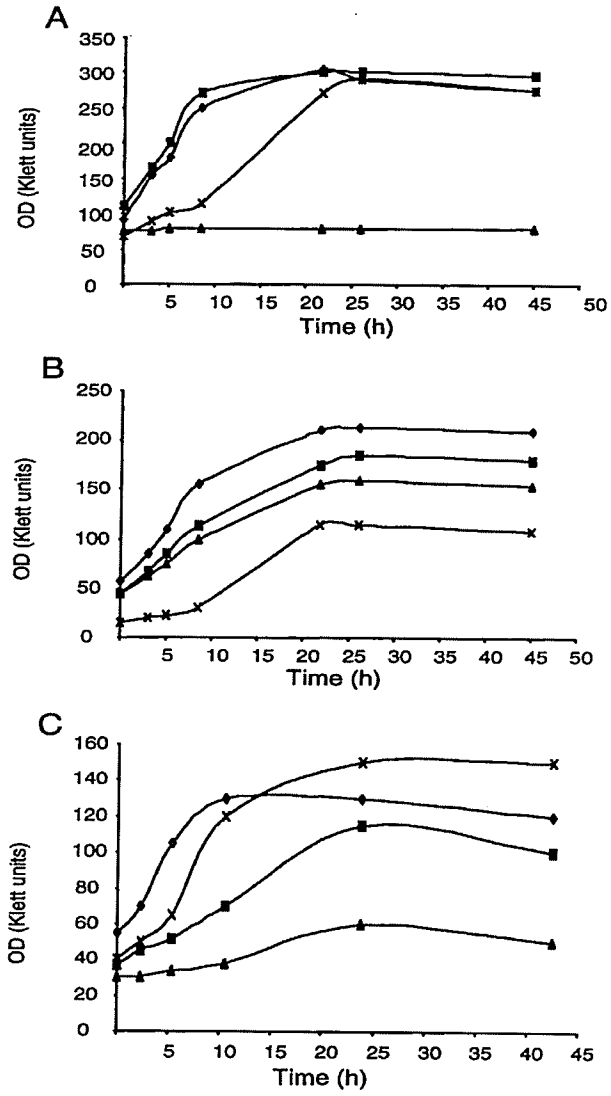

Fig. 2.9. Examples of salinity adaptation among investigated aerobic phototrophic strains isolated from Mahoney Lake. (A) Obligately halophilic strain ML6^T. (B) Strain ML3 is well adapted to fluctuating salinity, able to grow in both freshwater and saline media with small difference. (C) Halophilic strain ML35 with preference for high sulfate concentrations.

(♦), medium N1; (■), medium N2; (▲), medium N3; (×), medium N4.

Compared to *e. g.* freshwater *Sandaracinobacter sibiricus* (Yurkov and Gorlenko, 1990; Yurkov et al., 1992; 1997), which does not grow in media supplemented with 20 g NaCl/l, the newly isolated strains of APB clearly demonstrated broad salt tolerance. This ability may reflect an adaptation to an environment with a steep gradient of salinity. A similar tolerance of a range of salt concentrations was noted for other APB isolated from environments with variable salinity. Strains of *Erythrobacter litoralis* isolated from laminated marine mats on Texel island in the Netherlands (Yurkov and Van Gemerden, 1993), and strains of *C. bathyomarinum* recovered from a deep ocean black smoker plume waters (Yurkov et al., 1999), showed broad salt tolerance, indicating an adaptation to fluctuating salinities that may exist in a supralittoral zone and near deep ocean hydrothermal vents, respectively.

2.4.6. Phylogenetic analysis.

Based upon partial 16S rDNA sequences (>400 bases) all studied isolates belong to the alpha subclass of *Proteobacteria*. With similarity values ranging between 97 and 100% most isolates are closely related to species of the genera *Agrobacterium, Erythrobacter, Erythromicrobium, Porphyrobacter,* and *Sphingomonas* (Table 2.5). Whether these isolates should be regarded strains of described species or whether they represent new species should not be decided solely on the basis of molecular sequences. Other isolates are more distantly related (93-96% similarity) to described species of the genera *Paracoccus, Bosea, Sulfitobacter, Roseobacter,* and *Maricaulis* and these isolates are more likely to represent novel species and, eventually even novel genera if supported

Table 2.5. Phylogenetic relatedness of new isolates to described species of the α subclass of *Proteobacteria*.

Strain	Similarity (%) among each other	Similarity (%) to nearest neighbor	Nearest neighbor
ML1	NA	97.3	*Agrobacterium sanguineum*
ML10	NA	93.6	*Bosea thiooxidans*
ML14	NA	100	*Erythromicrobium ramosum*
ML19	NA	98.7	Genus *Porphyrobacter*
ML20	NA	97.2	Genus *Sphingomonas*
ML21	NA	97.6	*Erythromicrobium ramosum*
ML30	NA	95	Genus *Sulfitobacter*
ML35	NA	98.9	Genus *Sphingomonas*
ML36	NA	97.8	*Agrobacterium sanguineum*
ML45	NA	94	Genus *Roseobacter*
ML3, ML15	100	98.4	*Agrobacterium sanguineum*
ML42, ML43	100	94.5	Genus *Paracoccus*
ML4, ML31, ML32	100	98.8 98.4	*Porphyrobacter* *Erythrobacter litoralis*
ML22, ML23, ML34	100	99.8	*Agrobacterium sanguineum*
ML37, ML46, ML47	100	95	*Maricaulis maris*
ML6^T, ML16, ML17, ML18, ML29, ML33, ML38, ML40, ML44	100	95.6	Genus *Paracoccus*

NA, not applicable.

by other taxonomic criteria. The relationship of the lake isolates to members of the latter three genera is unexpected as they are of marine origin.

2.5 Concluding remarks.

The mixolimnion of the meromictic, saline Mahoney Lake harbors a diverse population of anoxygenic phototrophic bacteria. APB are distributed in all oxygenated zones to at least a depth of 5 m, and are present in high numbers (Table 2.1 and 2.2). Two purple nonsulfur photosynthetic isolates were also obtained from 5 m depth.

The APB population of Mahoney Lake consists of a wide variety of morphological forms (Table 2.2; Fig. 2.1-2.6), and the cytology of these cells revealed a gram negative cell wall organization. APB isolated from Mahoney Lake do not develop extensive photosynthetic ICM, which is characteristic of this group of bacteria (Yurkov and Beatty, 1998a; Shimada, 1995).

Different types of LH complexes were found in the new isolates, as indicated by absorption spectra (Fig. 2.8), including several presumably new types of LH complexes. Further investigation of the photosynthetic apparatus in these isolates may reveal how the protein environment around BChl *a* affects its absorption properties.

The majority of strains isolated from Mahoney Lake were shown to utilize a variety of organic substrates, similar to other aerobic phototrophs previously described. However, some strains were unable to grow on any single organic compound tested, whereas a complex organic nutrient such as yeast extract supported growth (Table 2.3). This is unusual for APB, and may indicate dependence on the organic nutrients (thought to be mainly complex, humic-like materials) available in the oligotrophic Mahoney Lake.

The majority of strains isolated have vitamin requirements or require as yet undetermined growth factors, and grow at high pH values as are found in Mahoney Lake (Table 2.3).

Mahoney Lake has vertical zones of variable salinity from the surface to the bottom. Different salt tolerances were established in strains isolated from different salinity concentrations. The majority of strains are adapted to the lake's salinity fluctuation, being able to grow in freshwater and in highly saline media (Fig. 2.9; Table 2.4). Perhaps because Mahoney Lake is a sulfate rich lake, most of the isolates grew best in sulfate enriched media (Table 2.4). The strains isolated from higher salt concentrations (5 m depth) grew best in highly halophilic medium N4 and did not grow in the absence of a salt supplement.

Similar to previously described species of the aerobic phototrophic and purple non-sulfur bacteria (Yurkov and Beatty, 1998a), new isolates belong to the α-subclass of *Proteobacteria* closely related to each other and to non-phototrophic representatives of this subclass. An interesting observation of our phylogenetic study is a demonstration of close relation of some strains purified from a meromictic lake to species obtained from different marine environments. Mahoney Lake is a relatively small lake, naturally and distantly isolated from the world ocean. Therefore, it is of interest to investigate the origin and evolution of new strains in the context of history and evolution of Mahoney Lake.

2.6. Acknowledgments.

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Chapter 3.

Isolation of tellurite- and selenite-resistant bacteria from hydrothermal vents of the Juan de Fuca Ridge in the Pacific Ocean.

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The material presented in Chapter 3 is derived equally from experiments completed by Christopher Rathgeber and Vladimir Yurkov. Coauthors contributing to this paper were Natalia Yurkova, who began the characterization of strains described herein and Erko Stackebrandt, who performed the phylogenetic analyis. This research was initiated in the lab of J. Thomas Beatty.

3.1. Abstract.

Deep ocean hydrothermal vent environments are rich in heavy metals and metalloids, and present excellent sites for the isolation of metal resistant microorganisms. Both metalloid oxide resistant, and metalloid oxide reducing bacteria were found. Tellurite and selenite reducing strains were isolated in high numbers from ocean water near hydrothermal vents, bacterial films, and from sulfide-rich rocks. Growth of these isolates in media containing K_2TeO_3 or Na_2SeO_3 resulted in the accumulation of metallic Te or Se. The minimum inhibitory concentration (MIC) of K_2TeO_3 ranged from 1500 μg/ml to greater than 2500 μg/ml, and the MIC of Na_2SeO_3 ranged from 6000 μg/ml to greater than 7000 μg/ml in ten strains. Phylogenetic analysis of four of these ten strains revealed that they form a branch closely related to members of the genus *Pseudoalteromonas,* within the γ-3 subclass of the *Proteobacteria.* All ten strains were found to be salt-, pH-, and thermotolerant. Metalloid resistance, as well as morphological, physiological and phylogenetic characteristics of newly isolated strains are described.

3.2. Introduction.

Se, a naturally occurring element, is essential for biological systems at low concentrations and toxic at higher levels. Under aerobic conditions Se is present in several redox forms including the elemental form Se(0), however, it exists predominantly in the high valence toxic and soluble forms, selenate (SeO_4^{2-}, +VI) and selenite (SeO_3^{2-}, + IV) (Conde and Sanz Alaejos, 1997). The roles of Se in the biosphere, both beneficial and detrimental, are gradually being determined (Daniels, 1996), and it is becoming evident that microorganisms play a major role in the global Se cycle. Reduction of

selenite to elemental Se (insoluble and non-toxic) was suggested to be used by various species of bacteria to eliminate the toxic character of this compound (Moore and Kaplan, 1992; Van Fleet-Stalder et al., 1997). Selenite is also involved in anaerobic respiration of several species (*Thauera selenatis, Sulfospirillum barnesii, Bacillus arsicoselenatis* and *Bacillus selenitireducens)* (Macy et al., 1993; Switzer-Blum et al., 1998).

About a quarter of a century ago, Te was considered almost as an exotic element and was treated with certain diffidence by the most serious chemists. The impressive number of publications on Te-compounds during the last few years shows that Te is now widely used in applied chemical reactions (Petragnani and Lo, 1998). The natural Te cycle, however, has not been investigated and the role of microbes in this process has not yet been elucidated. Te has not been shown as a biologically essential element thus far, and potassium tellurite in concentrations as low as 1 μg/ml is toxic to most microorganisms (Summers and Jacoby, 1977). In fact, tellurite has been employed as an antimicrobial agent in growth media (Summers and Jacoby, 1977), and prior to the development of modern antibiotics as a therapeutic agent for the treatment of leprosy, tuberculosis, dermatitis, cystitis and eye infections (Taylor, 1999).

Intrinsic low level resistance to tellurite has been reported for some Gram positive bacteria, however the mechanism of this resistance is still poorly understood (Summers and Jacoby, 1977). Resistance to tellurite in some Gram negative bacteria has been shown to be plasmid mediated (Summers and Jacoby, 1977). In *E. coli* the low-level basal resistance to tellurite is a result of a secondary function of the nitrate reductase (Avazeri et al., 1997). Resistance to extremely high levels of tellurite has been reported in members of the phototrophic purple non-sulfur bacteria family *Rhodospirilliacea* (Moore

and Kaplan, 1992; 1994; O'Gara et al., 1997) and in some members of the aerobic phototrophic bacteria, which have been shown to resist and reduce concentrations of tellurite as high as 2700 μg/ml (Yurkov et al., 1996). Recently a haloalkaliphilic archaeon, *Natronococcus occultus*, was also shown to be resistant to and reduce high levels of tellurite (Pearion and Jablonski, 1999).

Although the exact mechanism of toxicity of selenite and tellurite is not understood, the toxicity of these oxyanions has been attributed to their strong oxidizing activity, which may interfere with basic cellular process (Summers and Jacoby, 1977). Reduction of tellurite by bacteria results in black colonies on agar plates and a black culture in liquid media due to the accumulation of intracellular crystals of elemental Te (Moore and Kaplan, 1992; Taylor et al., 1988). When grown in the presence of high levels of sodium selenite cells accumulate intracellular deposits of the red amorphous elemental Se, giving cultures an orange to dark red color (Moore and Kaplan 1992; Sabaty et al., 2001).

There is a narrow concentration range between Se as an essential nutrient and as a toxic substance for animals and humans. While in some regions of the world the daily food intake is artificially supplemented with Se for health reasons, other regions are polluted with Se (Lauchli, 1993). In the San Joaquin Valley of California, bird malformations due to excess Se have been reported (Ohlendorf and Santolo, 1994). Chemical detoxification of metal- and metalloid-polluted sites is very expensive and often results in secondary effects on the environment. In contrast, biological solutions could be developed. Te is relatively rare in the environment, but it can be found at high concentrations near waste discharge sites. Native elemental Te is uncommon (an

abundance in the lithosphere of $2x10^{-7}$%), usually occurring in conjunction with elemental sulfur or as tellurides of lead, copper, silver, gold and antimony (Bagnall, 1975). The extraction of Te is difficult because of its low content in natural ores and Te compounds are usually obtained as by-products of metal refining processes (Klevay, 1976). Today, microbial bioremediation of toxic compounds and microbial concentration of metals from natural ores and from mining tailings with metal levels too low for smelting are becoming more popular (Madigan et al., 1997). However, it is currently estimated that only about 1% of microbial species have been described and obtained in laboratory culture. Thus a continuous search for new microbes with biotechnological and industrial potential is of high importance.

Volcanic and hydrothermal processes associated with the global mid-ocean ridge system support a potentially vast, complex ecosystem on and beneath the deep ocean floor. Deep ocean hydrothermal vent fluids possess a wide range of chemical compositions (Butterfield et al., 1994). These fluids have been found to be enriched in metal sulfides, which include iron, copper, calcium, silicon, zinc as well as metalloids (Butterfield et al., 1994; Feely et al., 1987). Whereas the average concentration of Te (550 fmol/kg) and Se (1.9 nmol/kg) in the ocean is relatively low (Lee and Edmond, 1985; Measures and Burton, 1980), their concentration around deep ocean vents is significantly higher. The measured values for Se in the particle fraction from the vents at Guaymas Basin and from 21°N, East Pacific Rise range from 15 to 103 nmol/kg (Von Damm et al., 1985a; 1985b). The concentration of Te and Se in sulfide rocks of the Galapagos Rift was measured as 111 µmol/kg and 569 µmol/kg, respectively (Knott et al., 1995). The high concentration of metals and metalloids in fluids and mineral deposits

surrounding the vents (Butterfield et al., 1994) may make them an ideal location for the isolation of metalloid resistant microorganisms. In this study, we report on the isolation, enumeration, and the characterization of major physiological properties of several strains that resist and reduce high concentrations of tellurite and selenite. These bacteria were recovered from samples taken in the vicinity of deep ocean hydrothermal vents located in the Main Endeavour Segment of the Juan de Fuca Ridge in the Pacific Ocean.

3.3. Materials and methods.

3.3.1. Collection of samples.

Samples were obtained in June, 1998 on the R/V Atlantis utilizing the deep-ocean submersible ALVIN at the Juan de Fuca Ridge main Endeavour Field in the Pacific Ocean (47.57'N, 129.05'W), from depths of 2000 to 2200 m. The vent plume water collected in Niskin bottles, bacterial film-like formations collected using a slurp gun and pieces of sulfide rocks were obtained to isolate metalloid reducing bacteria (Table 3.1).

3.3.2. Isolation and enumeration of metalloid resistant bacteria.

Isolation and enumeration of strains was performed using a solid (2% agar) medium (designated HM) that contained (g/l): NaCl, 20; KH_2PO_4, 0.3; NH_4Cl, 0.3; KCl, 0.3; $CaCl_2 \cdot 2H_2O$, 0.05; Na-acetate, 1; Na-malate, 0.3; yeast extract, 0.1. HM medium was supplemented with a mixture of vitamins ([per liter]: 20 μg of vitamin B_{12}, 200 μg of

Table 3.1. General characteristics of isolated strains.

Strain	Color and type of colonies without metal addition	Color of colonies isolated on media containing		Morphology (size[μm])	Site (depth of isolation)
		K_2TeO_3	Na_2SeO_3		
Te-1-1	Opaque, creamy	Black	NA	Short ovoid rod (0.7 × 1.0) or rod (0.8 × 2.8)	Bacterial-film-like community, Melarie Summit, Main Endeavor Field (~2,200 m)
Te-1-2	Transparent, colorless	Black	NA	Curved rod (0.9 × 2.1), vibrio (0.7 × 2.0), or short spirillum (0.7 × 3.5)	Same as for strain Te-1-1
Se-1-1-or	Opaque, creamy	NA	Orange	Short ovoid (0.9 × 1.4) or elongated (1.1 × 2.4) rods	Same as for strain Te-1-1
Se-1-2-or	Opaque, creamy	NA	Red-orange	Short ovoid (0.7 × 1.0) or elongated (1.0 × 2.4) rods	Same as for strain Te-1-1
Se-1-2-red^T	Opaque, creamy	NA	Bright red	Short (0.9 × 1.4) or slightly curved long (0.8 × 2.5) rods	Same as for strain Te-1-1
Se-1-3-red	Transparent, creamy	NA	Bright red	Short ovoid (1.2 × 1.8) or elongated (1.0 × 2.7) rods	Same as for strain Te-1-1
Te-2-1	Transparent, colorless	Black	NA	Vibrio (0.8 × 2.4) or short spirillum (0.6 × 5.3)	Water beneath Lobo Flange, Main Endeavor Field (~2,194 m)
Te-2-2^T	Transparent, colorless	Black	NA	Vibrio (0.6 × 1.9) or short spirillum (0.7 × 3.2)	Same as for strain Te-2-1
Se-6-1-or	Transparent, colorless	NA	Bright red	Short ovoid (0.7 × 1.2) or elongated (0.8 × 2.6) rods	Wash from sulfide rocks, Site Dante, Main Endeavor Field (~2,186 m)
Se-6-2-red	Transparent, creamy	NA	Dark red	Short ovoid (1.0 × 1.5) or elongated (0.7 × 2.3) rods	Same as for strain Se-6-1-or

NA, not applicable.

nicotinic acid, 80 μg of biotin and 400 μg of thiamine) and 1.0 ml per liter of a trace element solution (Drews, 1983). Filter sterilized cystein and methionine (both 0.3 mM), 5 ml of each per liter of medium, were added after autoclaving of the basal medium. For selection of selenite and tellurite resistant bacteria, 100 μg of either sodium selenite or sodium tellurite (from filter sterilized 10% solutions) per ml of medium were added. The pH was adjusted to 7.8.

3.3.3. Media used for subsequent cultivation.

Isolates were grown in medium D/O containing the following in g/l: KH_2PO_4, 0.3; $MgSO_4$, 0.5; NH_4Cl, 0.3; KCl, 0.3; $CaCl_2$, 0.05; Na-acetate, 1.0; yeast extract, 0.5; Casamino acids, 0.5; NaCl, 15.0. Media were adjusted to pH 7.8- 8.0 and supplemented with a mixture of vitamins: (per litre: 20 μg of vitamin B_{12}; 200 μg of nicotinic acid; 80 μg of biotin; and 400 μg of thiamine), and 2.0 ml per litre of a trace element solution (Drews, 1983).

Plates (supplemented with 2% agar) and liquid cultures were incubated aerobically in the dark at 28°C. Physiological and biochemical tests were performed using D/O medium unless otherwise specified.

3.3.4. Morphology and cytology.

The Gram test was done by the method of Gregersen (1978). The size and shape of cells were determined by phase-contrast and electron microscopy of cells from cultures grown in D/O and HM media (100 μg of metalloid oxide per ml). Cytology of cells was investigated from thin sections examined in an electron microscope. For thin sections,

the bacteria were embedded in Epon after fixation with 1% glutaraldehyde and 1% osmium tetroxide as described (Kellenberger et al., 1958).

3.3.5. Resistance to and reduction of metalloid oxides.

Strains were grown on D/O plates overnight and cells were transferred into HM medium to prepare a dense cell suspension. A loopfull of cell suspension was deposited on plates of HM medium (supplemented with an appropriate volume of either 10% K_2TeO_3 or 10% Na_2SeO_3) and incubated for up to 6 days at 30°C in the dark. Resistance was determined by the appearance of growth and reduction of oxyanions to their respective elemental forms by color of growth as indicator: grey to dark black in the case of tellurite reduction and orange to dark red in the case of selenite reduction. The MIC was defined as the lowest concentration of either K_2TeO_3 or Na_2SeO_3 that inhibited growth.

The viability of cells following exposure to high levels of either tellurite or selenite was determined after 5 days of incubation on plates containing 2000 μg/ml K_2TeO_3 or 2000 μg/ml Na_2SeO_3, by removing cells with a loop and streaking on D/O plates.

The ability to grow anaerobically using either K_2TeO_3 or Na_2SeO_3 as a terminal electron acceptor for anaerobic respiration was tested in screw cap tubes completely filled with HM medium containing appropriate concentrations of K_2TeO_3 or Na_2SeO_3. Optical density was measured using a Klett-Summerson photoelectric colourimeter, before and after incubation at 30°C for 10 days. Anaerobic growth was also tested on HM plates incubated in an anaerobic jar containing a CO_2+H_2 headspace (BBL GasPak), at 30°C for

14 days, as well as on modified HM plates where Na-acetate and malic acid were replaced by the addition of 1.0 g/l glucose.

3.3.6. Biochemical and physiological properties.

Physiological and biochemical tests were performed as previously described (Yurkov and Van Gemerden, 1993; Yurkov et al., 1994).

The ability to reduce nitrate was examined in partially filled tubes containing D/O medium with 0.1% KNO_3, incubated without shaking for 10 days. Following incubation, α-naphthylamine and sulfanilic acid were added to determine the presence of nitrite. If no nitrite was present, zinc dust was added to evaluate the presence of nitrate remaining in the medium. Tubes that contained inverted Durham vials were used to detect gas production (Pimenova et al., 1983).

To test for sugar fermentation, 0.1% of either glucose or fructose was added to D/O medium free of other organic carbon sources. Stationary tubes were incubated for 7 days, pH was measured to determine acid production and inverted Durham vials were used to detect gas production.

3.3.7. Phylogenetic analysis.

Extraction of genomic DNA, PCR-mediated amplification of the 16S rDNA and direct sequencing of the purified PCR product were carried out as described (Rainey et al., 1996). The 16S rDNA sequences were aligned with published sequences obtained from the EMBL Nucleotide Sequence Database (Cambridge, UK) and the Ribosomal Database Project (RDP), using the ae2 editor (Maidak et al., 1996). Evolutionary

distances were calculated and phylogenetic dendrograms were constructed as described (Jukes and Cantor, 1969; DeSoete, 1983). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 500 resamplings (Felsenstein, 1993).

The 16S rDNA sequences determined in this study were deposited in the EMBL database (Cambridge, UK) under the following accession numbers: strain Te-1-1 = AJ314843; Te-2-2^T = AJ314842; Se-1-2-or = AJ314844; Se-1-2-red^T = AJ314845.

3.4. Results and discussion.

3.4.1. Isolation and enumeration.

Samples of hydrothermal vent fluids were directly inoculated onto selective HM medium. Small pieces of sulfide rock samples were washed with sterile liquid base of HM medium in tubes using a vortex mixer, and the resultant suspensions used to inoculate selective (metalloid containing) plates. Plates were incubated at 30° C in the dark for a week. Tellurite resistant (black colored due to accumulation of elemental Te) and selenite resistant (orange to red colored due to accumulation of elemental Se) colonies were subsequently streaked on metalloid-free HM plates to complete purification of isolates. Pure strains were used for further investigation. The tellurite resistant (Te-2-1 and Te-2-2^T) and selenite resistant (Se-6-1-or and Se-6-2-red) strains were obtained from these samples (Table 3.1).

Bacterial film-like formations with surrounding vent fluid obtained at the Melarie Summit site were used to enumerate metalloid resistant/reducing bacteria present in this

community (Table 3.1). Bacterial aggregates were initially homogenized and later inoculated as decimal dilutions onto selective HM agar plates and onto HM agar plates without metal addition (controls). After incubation, the total number of cells that grew on metalloid-free medium was compared with the number of metalloid resistant cells that grew on HM(selenite) and HM(tellurite) medium. The numbers of CFU obtained from control plates showed up to 60000 CFU/ml of sample as white, creamy and colorless bacteria. The medium selective for tellurite resistant strains yielded black colonies and plates selective for selenite resistant strains gave rise to a variety of orange and red colored colonies (Fig 3.1). The results of this enumeration indicated that the number of tellurite resistant strains (1200 CFU/ml) in the samples was much smaller than the number of selenite resistant strains (the combined CFU/ml of all types of selenite reducing strains was 14240). We estimate that about 24% of strains able to grow on HM medium were selenite resistant and only about 2% of the population were tellurite resistant. However, we have two reservations. On the one hand, as an alternative to genuine dissimilatory metal reduction (an anaerobic process), some bacteria are thought to use metal oxide reduction for disposal of electrons (e.g., reoxidation of NADH, NADPH, $FADH_2$, reduced cyt or quinones), thereby maintaining an appropriate redox poise *in vivo* (Moore and Kaplan, 1992; 1994). Therefore, it is possible that some metalloid resistant strains grew on selective media only because growth was stimulated by the presence of the metalloid oxide. On the other hand, the newly isolated strains are phylogenetically related to described species of *Pseudoalteromonas* (see Section 3.3.7).

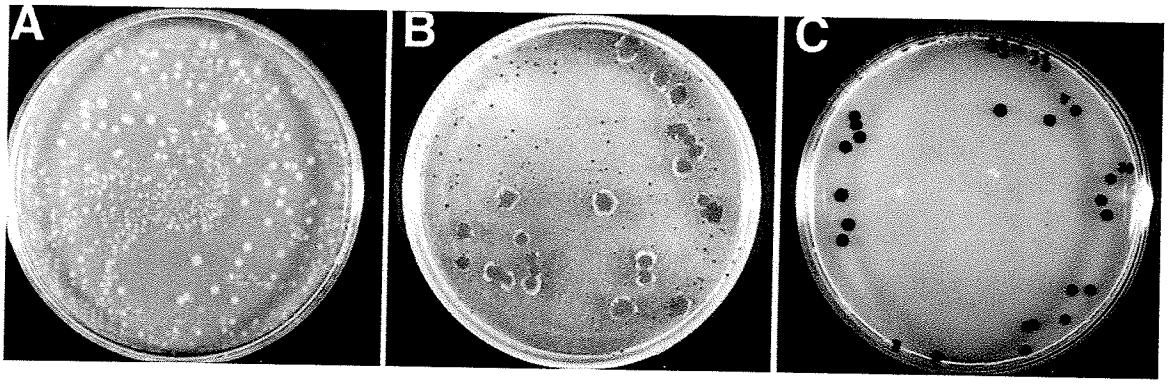

Fig. 3.1. Plates used for the enumeration of metalloid resistant/reducing bacteria in bacterial film-like formations taken from the Melarie Summit site, Main Endeavour Field. (A) Metalloid-free control plate. (B) and (C) plates containing 100 μg/ml Na_2SeO_3 and K_2TeO_3, respectively. Red and orange color of colonies (B) is due to accumulation of elemental Se and black appearance (C) is due to accumulation of elemental Te.

Some species of *Pseudoalteromonas* have been found to exhibit strong bacteriolytic activity against both Gram-positive and Gram-negative bacteria (Sawabe et al., 1998), which is a significant trait for bacteria living in oligotrophic environments. Because of this possible bacteriolytic activity in *Pseudoalteromonas* strains, it is possible that some Gram-positive as well as Gram-negative metalloid resistant bacteria did not form colonies on the selective plates. Tellurite resistant (Te-1-1 and Te-1-2) and selenite resistant (Se-1-1-or, Se-1-2-or, Se-1-2-red^T and Se-1-3-red) strains were isolated from the bacterial mat sample (Table 3.1).

3.4.2. Morphology and cytology.

Four strains (Te-1-1, Te-1-2, Te-2-1 and Te-2-2^T) isolated on tellurite-containing selective HM agar plates produced black, circular and lustrous colonies. Some of the strains isolated from Se-containing selective HM agar plates formed small orange to red-orange colonies (Se-1-1-or, Se-1-2-or, and Se-6-1-or), whereas others formed bright red to dark red colonies (strains Se-1-2-red^T, Se-1-3-red and Se-6-2-red). Growth of strains Te-1-1, Se-1-1-or, Se-1-2-or and Se-1-2-red^T on D/O agar plates without metalloid addition produced opaque creamy colonies. The colonies of strains Te-1-2, Te-2-1, Te-2-2^T and Se-6-1-or were transparent and colorless whereas the colonies of Se-1-3-red and Se-6-2-red were transparent and creamy (Table 3.1).

Colonies of two strains, Te-1-1 and Se-1-2-red^T, softened the agar and produced halos of clearing after 24 to 48h of incubation at 30°C indicating the production of an extracellular agarase. Extracellular agarase activity has been previously found in several bacterial strains from marine environments, including members of the genera

Alteromonas and *Pseudoalteromonas* (Holmstrom and Kjelleberg, 1999). It appears that the bacterial degradation of agar occurs by two mechanisms based on the specificity of the enzymes β- and α-agarase. Most of the reported agarolytic *Pseudoalteromonas* strains have been found to produce extracellular β-agarase, whereas a *P. agarolyticus* strain was reported to produce both α- and β-agarases (Holmstrom and Kjelleberg, 1999). Because strains Te-1-1 and Se-1-2-red^T are phylogenetically related to *Pseudoalteromonas*, it would be of interest to investigate which mechanism of agar hydrolysis is used by these strains.

Two distinct morphological types were identified when the isolated strains were grown in D/O medium without metalloid addition. One cluster (strains Te-1-1, Se-1-1-or, Se-1-2-or, Se-1-2-red^T, Se-1-3-red, Se-6-1-or and Se-6-2-red) included bacteria which have rod-shaped cells. The size of rods in culture was different and seemed to reflect the age of the cell. The cells divided by binary fission. The mature mother cell, which was an elongated rod of size 1.0 x 2.4 to 0.8 x 2.8 μm, depending on the strain, produced after division two short ovoid rods (sometimes almost coccoid cells) of size 0.9 x 1.4 to 0.7 x 1.9 μm. Motility was found in two strains (Te-1-1 and Se-1-2-red^T), whereas in the other five strains of this cluster motility was not observed (Table 3.1). Another cluster (strains Te-1-2, Te-2-1 and Te-2-2^T) was represented by bacteria with vibrio (0.6x1.9 to 0.8x2.4 μm) or short spirillum (0.7x3.2 to 0.6x5.3 μm) morphology. As is characteristic of vibrios and spirilla, all strains of this cluster are motile.

The Gram test and ultra-thin electron microscope sections indicated that all strains have a Gram-negative cell wall. Phase-contrast and electron microscopy showed that most of the strains, excluding three vibrio-spirillum type strains Te-1-2, Te-2-1 and Te-2-

2^T, produced capsular- or matrix-like extracellular compounds. Exopolysaccharide (EPS) producing strains are common within the genera *Pseudoalteromonas* and *Alteromonas*. Interestingly, *Alteromonas* sp. strain HYD-1545, isolated from tube worms, produces an EPS containing acidic sugars, which were demonstrated to have heavy metal binding properties (Vincent et al., 1994). This bacterium was suggested to be important for the survival of its host organism, which lives in an environment where the exposure to chemicals (e.g., toxic metalloid compounds) is high. It is generally thought that bacterial EPS may serve as protection against anti-bacterial substances, control bacterial attachment, protect against predation by protozoa, function as enhancers for nutrient uptake and reduce the diffusion of substances into and out of cells (Fletcher and Floodgate, 1973; Wrangstadh et al., 1986; Geesy et al., 1992; Vincent et al., 1994). However, the functional role as well as the chemical and structural composition of the extracellular excretions in our deep ocean strains await future studies.

Interesting changes in morphology were observed in strains that make a rod-shaped cluster depending on growth conditions and the presence of metalloid oxides (Fig. 3.2A, B, C, and D; strain Se-1-2-red^T as an example). In liquid medium these strains produced separate, short, ovoid and elongated rods, whereas on agar plates they tended to form chains (of up to 8 cells). The addition of selenite or tellurite to the HM medium provoked changes in cell morphology, the reduction of these oxides, accumulation of elemental Se or Te and the development of red-orange or black color in colonies, respectively.

During the first 48h of growth in the presence of metalloids, light orange (with Na_2SeO_3 added) or greyish (with Na_2TeO_3 added) colors developed. The cells in chains

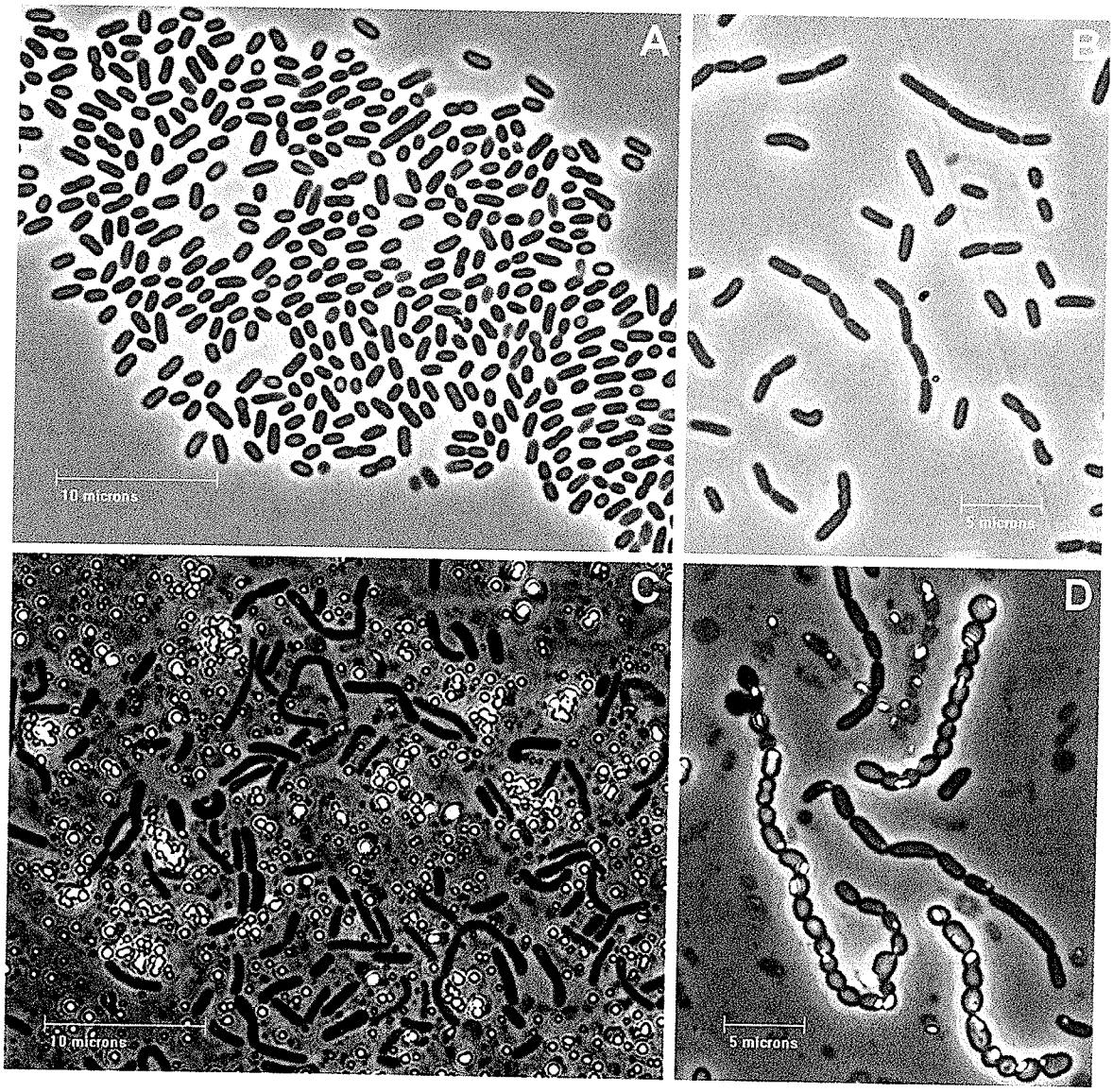

Fig. 3.2. Strain Se-1-2-red^T, phase contrast microscopy. (A) Cells grown in the absence of metalloids. (B) 48h-old culture grown in presence of tellurite. (C) and (D) 72h-old cultures grown in presence of selenite and tellurite, respectively. Most of Se globules were excreted from cells and present as cell-free light-refractile particles (C), whereas most of Te globules were still inside the cells (D).

became more elongated and a few light-refractile globules of Te or Se were seen inside as well as outside the cells. After 72h of growth, the colonies turned to bright orange/red (with Na_2SeO_3 added) or lustrous black (with Na_2TeO_3 added). At this time, most of the Se was observed as cell-free particles (Fig. 3.2C). However, after 72h in tellurite-reducing cultures, the cells in chains were much shorter, some almost rounded, with most of the Te inside the cells (Fig. 3.2D). The initial intracellular accumulation of Se and Te and the apparent later release of these particles by cells was also indicated in ultrathin electron microscope sections (Fig. 3.3). The three strains in the vibrio/spirillum cluster also reduced metalloid oxides, accumulated metalloid globules in the cytoplasm of the cell with subsequent release, but without noticeable changes in the morphology of cells.

Multiple detoxification processes may occur during selenite and tellurite reduction by microorganisms because elemental Se and Te have been described as deposits in the cytoplasm, in the periplasm, and outside the cell (Gerrard, 1974; Taylor et al., 1988; Lloyd-Jones et al., 1994; Suzina et al., 1995; Tomei et al., 1995; Yurkov et al., 1996; Losi and Frankenberger, 1997; Yamada et al., 1997; Yurkov and Beatty, 1998; Kessi et al., 1999; Taylor, 1999). According to Tomei et al. (1995), particles containing elemental Se found outside cells are released by cell lysis, whereas Losi and Frankenberger (1997) suggested that the reduction reaction occurs close to the membrane, possibly as a result of a membrane-associated reductase, and that the particles are rapidly expelled by a membrane efflux pump. Kessi et al. (1999) speculated that a vesicular mechanism of Se excretion occurs in *Rhodospirillum rubrum*. Therefore it will be a challenge to elucidate the mechanism of metal excretion in the metalloid reducers described above.

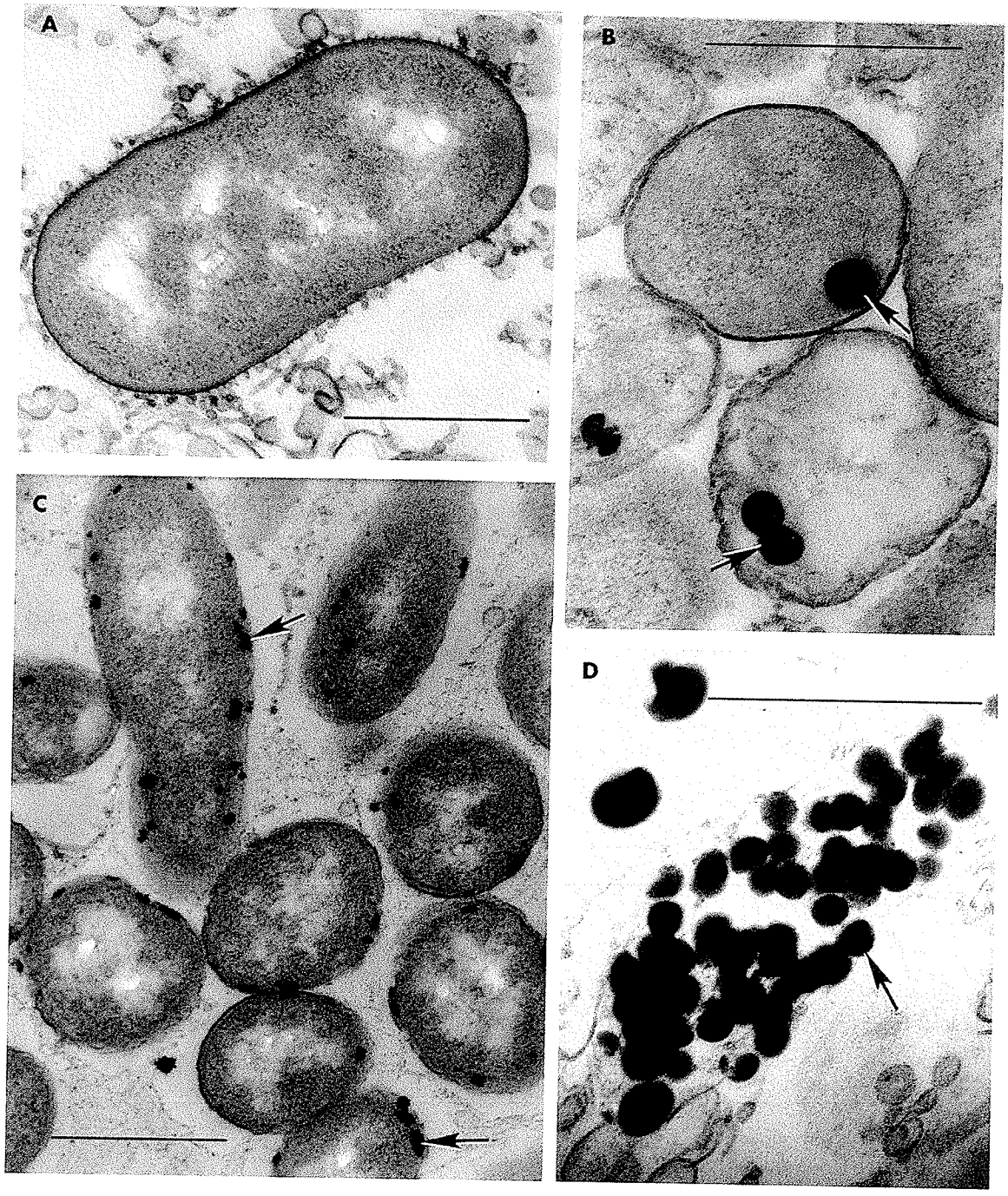

Fig. 3.3. Electron microscopy of ultrathin sections. Shown are: (A) strain Se-1-2-red^T, grown in metalloid-free medium; (B) intracellular localization of Se and (C) intracellular localization of Te, as a reduction product of selenite and tellurite, by strains Se-1-2-red^T (48h-old culture) and Te-1-1 (72h-old culture), respectively; (D) granules of Se released from cells of Se-1-2-red^T (48h-old culture). Bar: 0.5 μm.

3.4.3. Metalloid oxide resistance and reduction.

Although the strains described in this paper were isolated using a selective medium that contained either sodium tellurite or sodium selenite, all isolates revealed high resistance to both metalloid oxides. All strains, when grown on plates containing moderate levels of K_2TeO_3 (100 μg/ml) grew well and reduced TeO_3^{2-} to elemental Te, appearing as black colonies (Fig 3.1C), and as electron dense formations inside of cells in electron micrographs (Fig. 3.3C) (Moore and Kaplan, 1992; Lloyd-Jones et al., 1994).

The ability to resist high levels of K_2TeO_3 as well as the ability to reduce tellurite to elemental Te varied among strains as shown in Table 3.2. All strains except Te-1-1, Te-1-2, Se-1-2-or and Se-6-1-or were resistant to concentrations of K_2TeO_3 as high as 2500 μg/ml, although in all cases growth at concentrations above 2000 μg/ml was sub-optimal and the ability of cells to reduce large amounts of TeO_3^{2-} appeared to be hindered. Attempts to increase the concentration of K_2TeO_3 above 2500 μg/ml in the medium employed (pH 7.5 – 8.0) resulted in the formation of a white precipitate, and so higher concentrations were not tested.

All strains grew well on plates containing Na_2SeO_3 up to a concentration of 5000 μg/ml, and most strains showed high levels of SeO_3^{2-} reduction, resulting in accumulation of elemental Se at concentrations as high as 4000 μg/ml. Only strain Se-1-1-or was unable to reduce selenite in excess of 2000 μg/ml. Strain Te-2-2^T expressed the greatest resistance to SeO_3^{2-}, growing well and showing high levels of reduction up to 7000 μg/ml of Na_2SeO_3, the highest concentration tested.

Table 3.2. Comparative physiological characteristics of the strains isolated from the Juan de Fuca Ridge.

Characteristic	Result for strain				
	Te-1-1	Te-1-2	Te-2-1	Te-2-2^T	Se-1-1-or
Utilization of					
Acetate	+	+	+	+	W
Pyruvate	+	+	W	-	W
Glutamate	+	++	+	++	+
Butyrate	+	-	-	-	+
Citrate	+	-	-	+	+
Malate	-	-	-	+	+
Succinate	-	-	+	+	+
Lactate	-	-	+	+	+
Formate	-	-	-	-	-
Fructose	+	++	-	-	++
Glucose	+	+	+	++	++
Ethanol	-	-	-	-	+
Methanol	-	-	-	-	-
Yeast extract	++	++	++	++	++
Hydrolysis of					
Starch	+	+	-	-	+
Gelatin	+	-	-	-	+
Tween 60	++	++	++	++	+
Agar	++	-	-	-	-
Antibiotic sensitivity					
Chloramphenicol	+	+	+	+	+
Penicillin G	+	-	-	-	-
Streptomycin	+	+	+	+	-
Polymixin B	+	+	+	+	+
Tetracycline	+	+	-	-	-
Ampicillin	+	-	-	-	+
Kanamycin	+	+	+	+	-
Nalidixic acid	+	+	-	+	+
Reduction of metalloid oxyanions					
K_2TeO_3 (μg/ml):					
MIC	1500	2000	>2500	>2500	>2500
Max reduction	1300	1500	2000	2000	1300
Na_2SeO_3 (μg/ml):					
MIC	7000	6000	7000	>7000	5000
Max reduction	5000	5000	6000	>7000	2000

+, substrate is utilized, substrate is hydrolyzed or antibiotic sensitive; ++, substrate is utilized for very good growth; -, substrate is not utilized, substrate is not hydrolyzed, or antibiotic resistance; W, weak growth. Max reduction is the concentration at which growth and production of black coloration (in the case of strains grown in presence of K_2TeO_3) or red coloration (in presence of Na_2SeO_3) was optimal.

Table 3.2. Comparative physiological characteristics of the strains isolated from the Juan de Fuca Ridge (Continued).

Characteristic	Result for strain				
	Se-1-2-or	Se-6-1-or	Se-1-2-red^T	Se-1-3-red	Se-6-2-red
Utilization of					
Acetate	-	W	-	W	-
Pyruvate	-	W	+	+	-
Glutamate	++	++	+	+	+
Butyrate	W	-	+	+	+
Citrate	W	W	+	+	-
Malate	+	+	+	+	+
Succinate	+	+	+	+	-
Lactate	-	++	+	+	+
Formate	-	-	-	-	-
Fructose	++	++	++	++	+
Glucose	++	++	+	++	++
Ethanol	+	+	++	+	+
Methanol	-	-	-	-	-
Yeast extract	++	++	++	++	++
Hydrolysis of					
Starch	+	+	+	+	+
Gelatin	-	-	+	-	-
Tween 60	++	++	++	++	++
Agar	-	-	++	-	-
Antibiotic Sensitivity					
Chloramphenicol	+	+	+	+	+
Penicillin G	+	-	-	-	+
Streptomycin	-	+	+	-	-
Polymixin B	+	+	+	+	+
Tetracycline	+	+	+	-	-
Ampicillin	+	-	-	-	-
Kanamycin	+	+	+	-	+
Nalidixic acid	+	+	+	-	+
Reduction of metalloid oxyanions					
K_2TeO_3 (μg/ml):					
MIC	2000	1500	>2500	>2500	>2500
Max reduction	1300	1300	1300	1300	2000
Na_2SeO_3 (μg/ml):					
MIC	6000	6000	7000	6000	6000
Max reduction	4000	4000	5000	4000	4000

+, substrate is utilized, substrate is hydrolyzed or antibiotic sensitive; ++, substrate is utilized for very good growth; -, substrate is not utilized, substrate is not hydrolyzed, or antibiotic resistance; W, weak growth. Max reduction is the concentration at which growth and production of black coloration (in the case of strains grown in presence of K_2TeO_3) or red coloration (in presence of Na_2SeO_3) was optimal.

To determine the viability of cells after exposure to high concentrations of either K_2TeO_3 or Na_2SeO_3 (2000 μg/ml) for 5 days, cells were removed from plates and streaked onto fresh D/O plates free of metalloids (Moore and Kaplan, 1992). The strains Te-1-1, Te-2-2^T and Se-1-2-red^T (which all showed good growth and strong reduction at both 2000 μg/ml K_2TeO_3 and 2000 μg/ml Na_2SeO_3) continued to grow when transferred to the metalloid-free medium, with no visible black or red color, indicating that cells remain viable despite the accumulation of elemental Te or Se.

Strain Te-1-2 (which gave poor growth on 2000 μg/ml Na_2SeO_3) produced only a few colonies when transferred to the metalloid-free medium, indicating that although this concentration was toxic, some cells remained viable over the period of exposure.

Strain Se-1-1-or did not grow on plates containing 2000 μg/ml K_2TeO_3, but still reduced a small amount of tellurite to elemental Te, resulting in a grey appearance of the inoculum. When transferred to metalloid-free plates, these cells formed only a few isolated colonies indicating that some cells remained viable even after exposure to concentrations that inhibited growth.

The capability to use various oxyanions of Se as a terminal electron acceptor for anaerobic respiration has been described (Oremland, 1994; Laverman et al., 1995). To evaluate the ability of our strains for anaerobic respiration using either tellurite or selenite as an electron acceptor, tests were performed in both anaerobic tubes and on plates in an anaerobic jar. K_2TeO_3 and Na_2SeO_3 concentrations of 5, 100 and 1000 μg/ml were tested. None of the strains was capable of growth under anaerobic conditions. However, when grown in anaerobic tubes, strains Se-1-1-or and Se-1-3-red reduced a very small amount of tellurite to elemental Te, and strains Te-2-1, Te-2-2^T and Se-1-1-or reduced a small

amount of selenite to elemental Se. This indicates that metalloid reduction proceeds independently of growth while cells remain alive, and that reduction activity was retained by these strains even under sub-optimal conditions. On plates incubated in an anaerobic jar, no growth and no reduction of either K_2TeO_3 or Na_2SeO_3 was observed.

3.4.4. Diagnostic growth and physiological properties.

The major physiological properties of the isolates are summarized in Table 3.2. All strains were catalase and oxidase positive, and hydrolyzed Tween-60. Most strains hydrolyzed starch, and 3 strains hydrolyzed gelatin. Strains Te-1-1 and Se-1-2-or formed large clear halos on agar media resulting from agar hydrolysis.

All strains were capable of growth over a wide range of pH values. Five strains (Se-1-1-or, Se-1-2-or, Se-1-2-red^T, Se-1-3-red and Se-6-2-red) grew at all pH values tested from 5.0 to 11.0. Three strains (Te-1-1, Te-1-2, Te-2-2^T) were incapable of growth below pH 5.5, and two strains (Se-6-1-or and Te-2-1) grew over the slightly more narrow pH range of pH 5.5 to 10.0 (Table 3.2). Growth over a wide range of pH values might be indicative of an adaptation to the vicinity of hydrothermal vents, where low pH vent fluids mix with more alkaline surrounding waters, creating steep gradients in pH (Feely et al., 1987). The isolates also grew over a wide range of temperatures (Table 3.2), with most strains growing at temperatures from 5°C to 45°C. Again this could indicate an adaptation to the environmental conditions at the site of isolation, where high temperature fluids mix with cold surrounding waters creating steep temperature gradients (Feely et al., 1987).

All strains grew over a wide range of NaCl concentrations (up to 10%). Strain Se-1-1-or was capable of growth at 15% NaCl, and strains Se-1-3-red and Se-6-2-red grew in the presence of 20% NaCl, the highest concentration tested. All strains required NaCl concentrations $\geq$0.5% for growth. Strain Se-1-2-or showed the strongest requirement for salt, not growing below 1.5% NaCl (Table 3.2). Tolerance for high salt concentrations is not surprising as chloride concentrations in hydrothermal fluids has been reported at 30 – 200% of the levels found in the surrounding sea water (Butterfield et al., 1994) and high numbers of halotolerant bacteria in samples collected from the Endeavour segment of the Juan de Fuca Ridge have been reported (Kaye and Baross, 2000).

The isolates utilized a wide variety of organic compounds as the sole source of carbon for aerobic heterotrophic growth. The best carbon sources for most of the strains were glutamate, glucose and fructose. Anaerobic growth was not observed, although strain Se-6-1-or produced acid from glucose, and strains Te-1-2, Se-1-2-or and Se-6-1-or produced acid from fructose under microaerophilic conditions. Nitrate was not reduced by any of the strains. None of the strains required vitamins, although strain Te-2-2^T showed slightly reduced growth in media lacking thiamine. The ability to utilize a wide range of organic carbon sources and the lack of growth factor requirements may represent adaptations to the relatively oligotrophic waters found in the deep ocean, where less favorable nutrients must be used when more favorable nutrient sources become depleted.

The strains showed different responses to antibiotics (Table 3.2), varying from strain Te-1-1 which was susceptible to all antibiotics tested, to strain Se-1-3-red which was resistant to penicillin G, streptomycin, tetracycline, ampicillin, kanamycin and nalidixic acid.

3.4.5. Phylogenetic analyses.

16S rDNA phylogenetic analyses were performed on four representative strains: Te-1-1, Te-2-2^T, Se-1-2-or and Se-1-2-red^T. The sequences, covering 1143 (Se-1-2-red^T), 1468 (Te-2-2^T), 1500 (Se-1-2-or) and 1504 (Te-1-1) nucleotides, were compared among each other and with those of members of the class *Proteobacteria*. These four isolates share high 16S rDNA sequence identities, ranging between 99.7 and 99.9%; similarly high values are found for these isolates and the majority of type strains of species of the genus *Pseudoalteromonas,* a member of the γ-3 subclass of the *Proteobacteria*, which is composed of obligately marine, Gram-negative, obligately aerobic straight or curved rod shaped bacteria (Gauthier et al., 1995).

The *Pseudoalteromonas* genus contains several distinct phylogenetic clusters (Fig. 3.4), one of which embraces the four isolates as well as *P. carragenovora*, *P. espejana*, *P. atlantica*, *P. undina*, *P. elyakovii*, *P. distincta*, *P. nigrificans*, *P. haloplanctis*, *P. antarctica P. citrea* and some taxonomically invalid species. These organisms share higher than 98.5% sequence similarity, which explains the low statistical significance of the majority of branching points, as indicated by mostly low bootstrap values.

The high degree of sequence similarity among the majority of *Pseudoalteromonas* species makes it difficult to evaluate the taxonomic status of the four isolates. As demonstrated with *P. elyakovii* and *P. distincta* two different species may share 100% 16S rDNA identity (Sawabe et al., 2000).

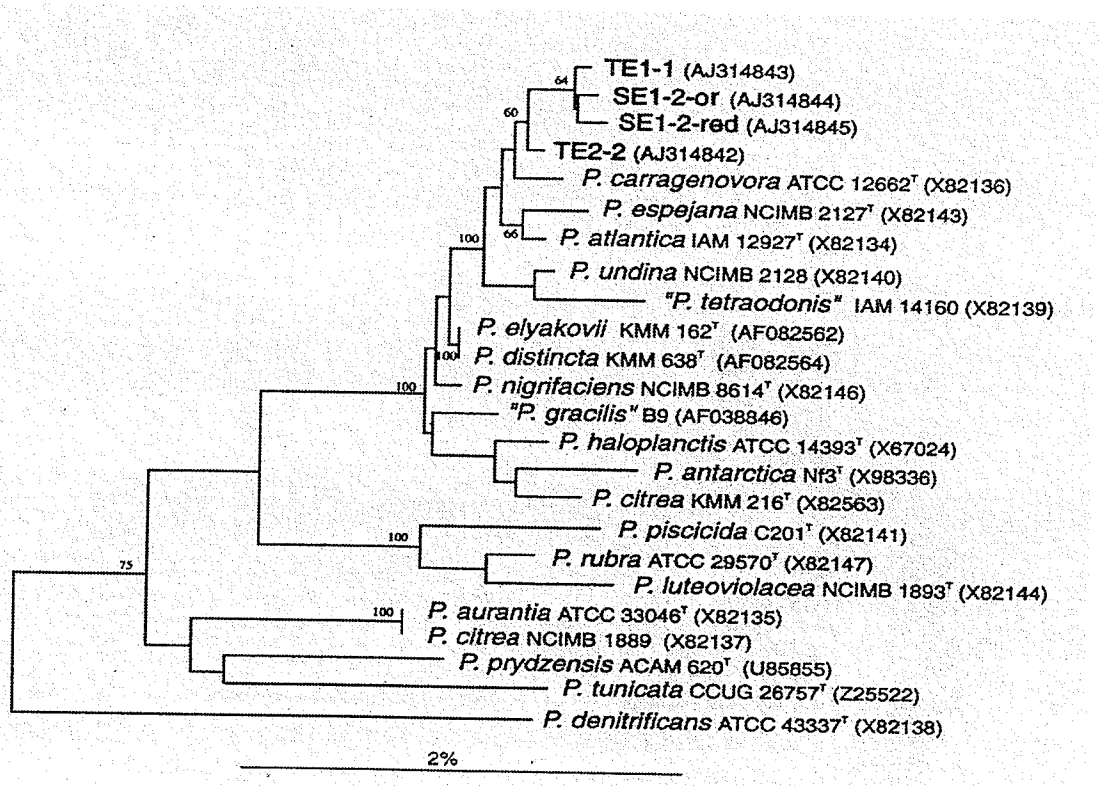

Fig. 3.4. Unrooted tree showing the phylogenetic position of four selected strains among members of the obligately marine genus *Pseudoalteromonas*, within the γ-subclass of the *Proteobacteria*.

3.5. Concluding remarks.

The vicinity of deep ocean hydrothermal vents along the Main Endeavour segment of the Juan de Fuca Ridge harbors a large number of bacteria resistant to high concentrations of Te and/or Se oxides. This high level of resistance could represent adaptations to the varied dissolved metal and metalloid ions present in hydrothermal vent fluids. Our results show that strains isolated from this extreme environment are resistant to very high concentrations of the toxic Te and Se oxyanions, and reduce them to less toxic elemental forms.

Four of the ten isolates were shown to be closely related to members of the genus *Pseudoalteromonas* on the basis of 16S rDNA sequence analyses. The major physiological properties of strains isolated in this study are in agreement with those described for the genus *Pseudoalteromonas* (Gauthier et al., 1995), although no member of this genus has yet been reported to resist or reduce oxyanions of either Te or Se. In contrast, members of the genus *Pseudoalteromonas* were described as having weak or irregular catalase activity (Gauthier et al., 1995), whereas our isolates all vigorously released O_2 when exposed to hydrogen peroxide, indicating a strong catalase activity.

The ability to reduce toxic Te or Se oxyanions to their less toxic elemental forms could play an important future role in bioremediation of highly polluted effluents from industrial and mining operations. In addition, bacteria capable of reducing tellurite and selenite could prove useful in the applied biometallurgy of Te and Se, rare and expensive metals used extensively for their properties as semi-conductors.

3.6. Aknowledgements.

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Chapter 4.

Roseicyclus mahoneyensis gen. nov., sp. nov., an aerobic phototrophic bacterium isolated from a meromictic lake.

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The material presented in Chapter 4 is derived primarily from experiments completed by Christopher Rathgeber. Coauthors contributing to this paper were Natalia Yurkova, who began the characterization of the strains described herein; Erko Stackebrandt, who performed the phylogenetic analysis; Peter Schumann, who performed the G+C determinations and Vladimir Yurkov, who guided the project. This research was initiated in the lab of J. Thomas Beatty.

4.1. Abstract.

Eight strains of Gram negative bacteria able to form ring-like cells were isolated from Mahoney Lake, a meromictic lake in south central British Columbia, Canada. All strains are pink-purple and contain bacteriochlorophyll *a* incorporated into the light harvesting I and II and reaction center pigment protein complexes. Growth does not occur anaerobically under illuminated conditions, and so these strains are obligately aerobic, which prompts us to designate them as members of the aerobic phototrophic bacteria. Physiological characterization revealed that these isolates share a similar tolerance to high levels of salinity and pH as is expected of bacteria from a highly saline lake, however, these strains exhibit marked differences in their ability to utilize organic substrates for aerobic heterotrophic growth. 16S rRNA sequence analysis shows these strains are closely related to the non-phototrophic genera *Octadecabacter* (92.0–92.9%) and *Ketogulonicigenium* (92.2–92.6%), and the aerobic phototrophs *Roseivivax* (92.2–92.9%) and *Roseovarius* (91.7–92.4%) within the α-subclass of the *Proteobacteria*. The DNA G+C base composition was 66.2 mol%. The unusual LH complex II, distinct morphological features and physiological traits of these strains as well as phylogenetic data, support the proposal of the new genus and species *Roseicyclus mahoneyensis* with ML6^T as the type strain.

4.2. Introduction.

Mahoney Lake is a meromictic saline lake located in the south central region of British Columbia, Canada. Interesting features of this lake include a sharp chemical discontinuity at the chemocline and high concentrations of Na^{+}, Ca^{2+}, Mg^{2+}, and SO_4^{2-}, leading to Mahoney Lake's classification as a sodium sulfate dominated lake (Northcote and Halsey, 1969; Hall and Northcote, 1986). The primary focus of most previous microbiological investigations of Mahoney Lake has been on the extremely dense population of the purple sulfur bacterium *Amoebobacter purpureus* (Overman et al., 1991; 1994; 1996). However strains of the purple sulfur bacterium *Thiocapsa roseopersicina*, the purple non-sulfur bacterium *Rhodobacter capsulatus*, and the green sulfur bacteria *Chloroherpeton thalasium* and *Prosthecochloris aestuarii* have also been isolated from the chemocline (Overman et al., 1991). In 1997 thirty-three aerobic BChl containing strains were isolated from the oxic mixolimnion, between the surface and 5 m depth. These strains included both purple non-sulfur and aerobic anoxygenic phototrophic bacteria, and exhibited a variety of interesting spectral absorption properties and morphologies (Yurkova et al., 2002).

Aerobic bacteria that contain BChl, commonly known APB, are a relatively recently discovered and taxonomically diverse group. The primary distinguishing features of the APB are the presence of BChl incorporated into LH and RC complexes, the relatively low level of photosynthetic units per cell, inhibition of BChl synthesis by light, inability to grow phototrophically under anaerobic conditions, a high mid-point potential of the RC primary electron carrier, and an abundance of carotenoid pigments (Yurkov and Beatty, 1998; Rathgeber et al., 2004).

The first reported member of the APB, *Erythrobacter longus*, is an orange pigmented rod shaped bacterium that was isolated from the Bay of Tokyo some 20 years ago (Shiba et al., 1979; Shiba and Simidu, 1982). Since that time other APB displaying a wide range of morphologies have been described. Members of the APB have been shown to produce typical rod shapes, elongated rods displaying thread-like cells and branching, ovoid cells, coccoid cells (Yurkov and Beatty, 1998), and one highly pleomorphic member of this group *Citromicrobium bathyomarinum* (Yurkov et al., 1999) has been described. Although several vibrioid and spirillum shaped genera and species have been described for the purple non-sulfur bacteria (Imhoff, 2001), no vibrioid species have been described as members of the APB.

In the current study we describe eight closely related vibrioid strains from Mahoney Lake as new members of the APB.

4.3. Materials and methods.

4.3.1. Strains and cultivation.

Strains ML6^T, ML16, and ML18 were isolated and cultivated on a rich organic medium, Medium N1 containing 15 g l^{-1} Na_2SO_4, and strains ML33, ML38, ML39, ML40 and ML44, on Medium N4 containing 50 g l^{-1} Na_2SO_4 (Yurkova et al., 2002). These media were used for all of the following experiments unless otherwise noted. Strains were kept on agar plates of the above media at 4°C for up to two months for short term storage. Long term storage was achieved by freezing at -70°C a thick cell

suspension in the above mentioned media supplemented with 30% glycerol as a cryoprotectant.

4.3.2. Morphological and cytological tests.

Cell size, shape and motility of late-log phase cultures were determined by phase contrast light microscopy. Electron microscopic negative stains were performed by staining cells with 1.0% aqueous uranyl acetate. For thin sections, the bacteria were embedded in Epon after fixation with 1.0% glutaraldehyde and 1.0% osmium tetroxide as described (Kellenberger et al., 1958).

4.3.3. Physiological and biochemical tests

The utilization of soluble organic substrates, Tweens, gelatin and starch, ability to grow at different pH levels, ability to ferment sugars and reduce nitrate, catalase and oxidase production and determination of antibiotic sensitivity were determined as previously described (Yurkov and Van Gemerden, 1993; Yurkov et al., 1994). Growth at differing salinities was tested in tubes containing the above described media with concentrations of NaCl or Na_2SO_4 of: 0, 0.5, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0 or 14.0%, incubated aerobically on a test-tube rotor at 30°C. Photoheterotrophic growth under anaerobic conditions was tested in screw-capped test tubes and in agar deeps containing the above described media, a basal medium designed for purple sulfur bacteria (Imhoff, 1988) containing H_2S or $Na_2S_2O_3$ and CO_2 with or without acetate, or a basal medium for purple non-sulfur bacteria (Imhoff, 1988) containing acetate, malate or succinate as the

sole source of organic carbon. Tubes were incubated at 30°C with tungsten filament lamp illumination of about 30 microeinsteins m^{-2} s^{-1}.

4.3.4. Pigment analysis.

Spectral absorption measurements were performed on strains grown under aerobic conditions in liquid culture. Cell samples were collected by centrifugation and resuspended in 125 µl of 10 mM Tris-HCl buffer (pH 7.8) and added to 375 µl of a 30% BSA solution (ICN Biomedicals Inc.) to reduce light scattering. Absorption spectra were recorded using a Hitachi U-2010 spectrophotometer.

4.3.5. G+C content determination.

The DNA base composition was determined by HPLC (Tamaoka and Komagata, 1984) of nucleotides obtained according to Mesbah and Whitman (1989).

4.3.6. 16S rDNA sequence analysis.

Extraction of genomic DNA, PCR-mediated amplification of the 16S rDNA and direct sequencing of the purified PCR product were carried out as described (Rainey et al., 1996). The sequence reaction mixtures were electrophoresed using a model 373A automatic DNA sequencer (Applied Biosystems). The partial 16S rDNA sequences were aligned with published sequences obtained from the EMBL Nucleotide Sequence Database (Cambridge, UK) and the Ribosomal Database Project (RDP), using the ae2 editor (Maidak et al., 1996) and similarity values determined.

4.3.7. Nucleotide sequence accession numbers.

The 16S rDNA sequence determined for strain ML6^T has been deposited in the EMBL database (Cambridge, UK) as a representative of this group, under the accession number AJ 315682.

4.4. Results and discussion.

4.4.1. Isolation.

The strains examined in this taxonomic description were isolated from the meromictic Mahoney Lake in south central British Columbia, Canada, in October 1997. Details of the isolation and enumeration of these bacteria were given by Yurkova *et al.* (2002).

4.4.2. Culture properties.

All strains formed small ~ 2 mm diameter pink-purple to purple colonies on the surface of agar media. In liquid media, grown under aerobic conditions, cultures appear pink-purple after 24 hours, becoming more purple with age.

Growth did not occur anaerobically under either light or dark conditions, and light was not required for growth under aerobic conditions, which led us to designate them as APB (Yurkov and Beatty, 1998).

4.4.3. Morphology and cytology.

Morphology was examined in log phase cells grown in rich organic medium (described above) under aerobic conditions. Strains ML6^T, ML16, ML18, ML33, ML38, ML39, ML40 and ML44 showed a similar morphology, which varied from elongated rods to slightly curved rods to vibrioid, almost cyclical cells (Fig. 4.1a). The dimensions of elongated rods were 0.6x2.6 μm and of vibrioid cells 0.6-0.7x2.7-3.0 μm. This morphology is similar to that of the purple non-sulfur bacterium *Rhodocyclus purpureus* (Pfennig, 1978), although our isolates have pointed ends, differing from cells of *R. purpureus*, which have flat to rounded ends.

Electron microscopy of negatively stained cells shows the pointed regions at the poles to be electron dense (Fig. 4.1b), and electron microscopy of thin sections indicates that these polar zones are actually due to an enlarged periplasmic space found in both rod shaped and vibrioid shaped cells (Fig. 4.1c, d). The appearance is similar to that seen in *Rhodospirillum tenue* (Pfennig, 1969) although the significance of these polar, periplasmic structures is unclear. Polar localization of proteins critical for cell division, chromosome partitioning and cell cycle control in *Escherichia coli, Bacillus subtilis,* and *Caulobacter crescentus* have been described. Bacterial polarity seems to play a critical role in cell structure and life cycles (Lybarger and Maddock, 2001).

The above mentioned strains are all non-motile. Cell division in strain ML6^T occurs by way of symmetric and asymmetric constrictions.

The Gram negative structure of the cell wall is confirmed by the electron microscopic thin sections, but ICM formations, of the type usually found in true anoxygenic phototrophs, are not observed, which is typical for the APB (Fig. 4.1c,

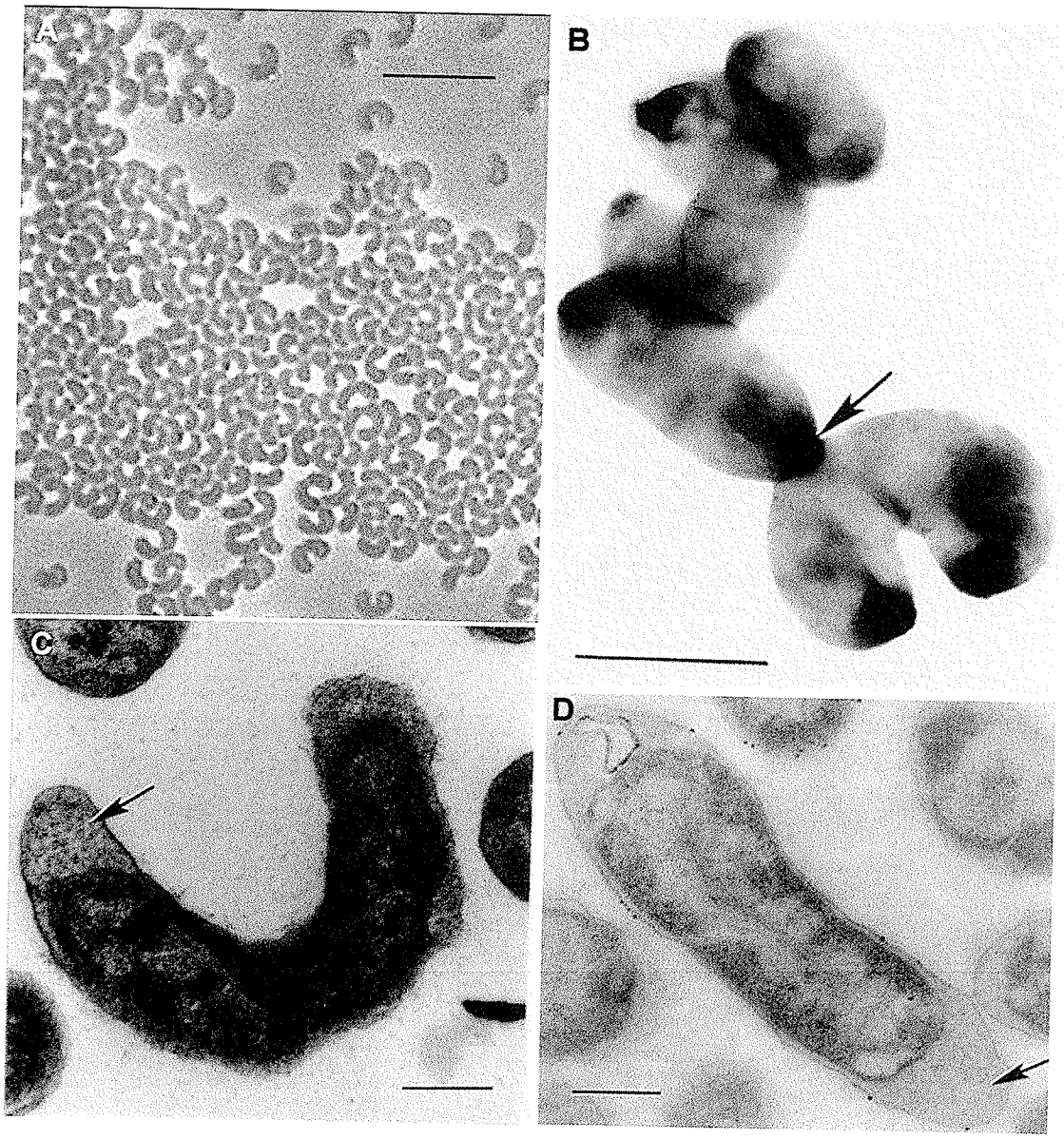

Fig. 4.1. (A) Phase contrast microscopy of strain ML6^T showing cyclical shaped cells. (B) Negative stained cells with polar dark coloration of pointed ends. (C, D) Ultra-thin sections of cells show polar periplasmic modifications (confirming polar staining in B; indicated by arrows). Bars: (A) 5 μm (B) 0.5 μm, (C, D) 0.25 μm.

d). Cells contain electron clear inclusions presumably due to storage of poly-beta-hydroxyalkoanates.

The unusual morphological characteristics of these strains, ranging from rod through vibrioid to cyclical shape, and pointed periplasmic space have not been previously reported in APB (Yurkov and Beatty, 1998). Thus these isolates are an exciting new addition to this already morphologically diverse group.

4.4.4. Photosynthetic apparatus.

Absorption spectra for the representative strain ML6^T are shown in Fig. 4.2, which as for all the strains in the present study show *in vivo* BChl *a* peaks at 805 to 806 nm and at 870 to 871 nm. The 870 to 871 nm peak is indicative of the LH I, and the peak at 805 to 806 nm is indicative of a peripheral LH II. This unusual organization of the photosynthetic apparatus, where the LH II complex has only one peak at approximately 805 nm has been found in only two genera of the APB thus far, namely *Roseobacter* (Shiba, 1991) and *Rubrimonas* (Suzuki et al., 1999), both of which have morphological and physiological characteristics quite distinct from our isolates. The presence of a relatively small RC peak located in the region of 800 nm is masked by the strong LH II peak at 805 nm, as indicated in other experiments in which the RC and LH complexes were purified from the photosynthetic membranes of ML6^T (Rathgeber et al., unpublished).

These isolates lack absorption peaks in both the 805 and 870 nm regions when grown aerobically in the presence of continuous illumination (as shown for strain ML6^T

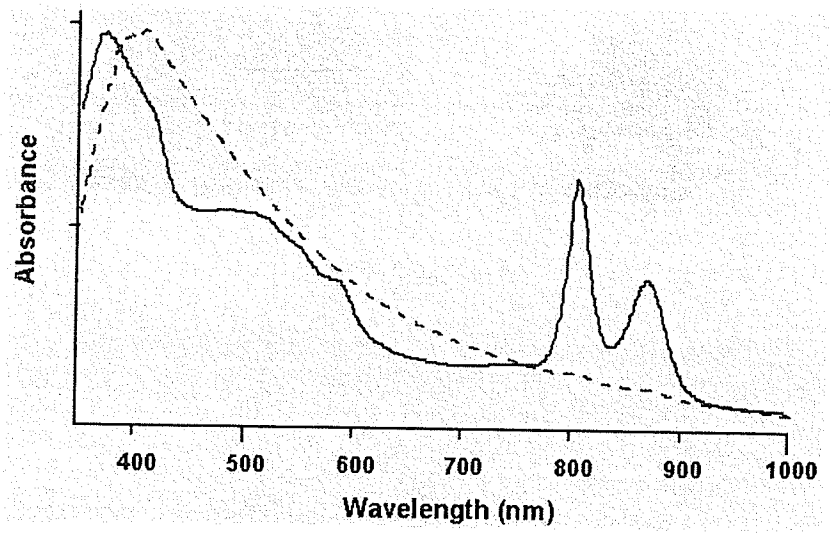

Fig. 4.2. Absorption spectra of strain ML6^T intact cells grown aerobically in the dark (solid line) show peaks at 805 and 870 nm corresponding to BChl incorporated into LH II and LH I complexes, respectively. These peaks are reduced or absent in cells grown aerobically in presence of continuous illumination (dashed line).

in Fig. 4.2). This trait further confirms the identification of these strains as members of the APB. All strains show similar carotenoid peaks at approximately 408 and 484 nm.

4.4.5. Biochemical and physiological data.

A variety of physiological characteristics is presented in Table 4.1. All of the isolates produce both catalase and cyt *c* oxidase.

The group of strains represented by ML6^T shows an absolute requirement for saline conditions, unable to grow at concentrations of NaCl or Na_2SO_4 (or presumably another compatible solute) less than 0.5%. The upper salinity limits for all of the strains is similar. All strains grow up to 10% Na_2SO_4 and the upper limit for NaCl is between 6 and 10%, differing between strains.

All strains tolerate a wide range of pH values and grow between pH 6.0 and 11.0, the highest value tested.

In a previous study, thirty-three Mahoney Lake isolates were categorized into three groups based on their ability to utilize organic substrates as sole sources of carbon and energy (Yurkova et al., 2002). The largest group was made up of strains that were able to utilize a wide range of substrates. A second group contained strains with very restricted metabolic abilities, utilizing only a limited number of organic substrates. A third and final group was made up of strains that did not grow in any media containing only single organic compounds, because they require a complex organic source such as yeast extract (Yurkova et al., 2002). The strains described herein fall into all three groups previously described, as shown in Table 4.1, indicating that the ability to utilize organic substrates is not an adequate taxonomic marker for the APB isolated from Mahoney

Table 4.1. Comparative physiological characteristics of the aerobic phototrophic strains isolated from Mahoney Lake and close phylogenetic relatives, *Roseobacter litoralis*, Och 149^T, *Roseivivax halodurans*, Och 239^T and *Roseovarius tolerans*, EL-172^T.

Characteristic	Strain										
	ML6^T	ML16	ML18	ML33	ML38	ML39	ML40	ML44	Och 149^T	Och 239^T	EL-172^T
Growth at											
4°C	-	-	-	-	+	-	-	-	+		+
10°C	+	-	-	+	+	+	+	+	+		++
28°C	++	++	++	++	++	++	++	++	+	+*	++
37°C	+	-	-	+	+	+	+	+	-		+
45°C	-	-	-	-	-	-	-	-	-		-
Growth at pH											
5.5	-	-	-	-	-	-	-	-	-		-
6.0	++	++	++	++	++	++	++	++	-		-
7.0	++	++	++	++	++	++	++	++	-		+
8.0	++	++	++	++	++	++	++	++	+	+*	+
9.5	++	++	++	++	++	++	++	++	+		NA
10.0	++	++	++	++	++	++	++	++	-		NA
11.0	++	++	++	+	+	+	+	+	-		NA
Utilization of											
Acetate	+	+	+	-	-	-	W	-	+	+	+
Pyruvate	+	+	+	-	+	-	W	W	+	+	+
Glutamate	+	+	+	-	+	-	-	W	+	+	+
Butyrate	-	+	-	-	-	-	-	-	-	+	+
Citrate	+	-	+	W	+	+	+	+	+	+	-
Malate	+	+	+	-	+	-	-	-	+	+	+
Succinate	+	+	+	-	+	-	-	W	+	+	+
Lactate	+	+	+	-	+	-	W	+	NA	+	-
Formate	-	-	-	-	-	-	-	-	NA	NA	NA
Fructose	++	++	++	-	+	+	+	+	NA	+	+
Glucose	++	++	++	-	+	+	-	+	+	+	-
Ethanol	-	-	-	-	-	-	-	-	NA	-	NA
Methanol	-	-	-	-	-	-	-	-	-	-	-
Yeast extract	++	++	++	+	++	++	++	++	+	+	+
Hydrolysis of											
Starch	+	+	NG	-	-	-	-	-	-	NA	-
Gelatin	+	+	+	+	+	+	+	+	+	-	-
Tween 60	-	-	-	-	+	NG	+	+	+	NA	NA
Antibiotic Sensitivity											
Chloramphenicol	+	+	+	+	+	+	+	+	+	+	+
Penicillin G	-	-	-	-	-	-	-	-	+	-	+
Streptomycin	+	+	-	-	-	-	-	-	+	+	+
Polymixin B	+	+	+	+	+	+	+	+	+	NA	-
Tetracycline	+	+	+	+	+	+	+	-	NA	-	+
Ampicillin	-	-	-	-	-	-	-	-	NA	NA	NA
Kanamycin	+	+	-	-	-	-	-	-	NA	NA	NA
Nalidixic acid	-	-	-	-	-	-	-	-	NA	NA	NA

+, substrate is utilized, substrate is hydrolysed, vitamin required or antibiotic sensitive; ++, substrate is utilized for very good growth; -, substrate is not utilized, substrate is not hydrolysed, vitamin is not required or antibiotic resistant; W, very weak growth; NG, no growth; NA, not available; *, only optimum temperature and pH have been published (Suzuki et al., 1999).

Lake, where even highly similar and highly related strains show great differences in organic carbon preference.

The isolates described in this paper cannot be repeatedly transferred and cultivated on minimal media in the absence of yeast extract. This indicates that they require an as yet unknown growth factor present in yeast extract. Other strains from Mahoney Lake have been shown to be dependant on vitamin B12 and/or biotin (Yurkova et al., 2002), however the addition of these vitamins to minimal media was not sufficient to allow the successive cultivation of these isolates.

As is common for the APB, there was variable sensitivity to antibiotics (Table 4.1). Again this trait does not appear to be a useful taxonomic marker, as highly similar, highly related strains show markedly different responses to the antibiotics tested.

4.4.6. DNA composition and phylogenetic analysis.

Based on the analysis of almost complete 16S rRNA gene sequences (>1430 nucleotides), strain ML6^T is a member of the α-3 group of the *Proteobacteria*, within the *Roseobacter* clade (Fig. 4.3). Based upon analysis of the algorithm of De Soete (1983), neighbour joining and maximum likelihood, strain ML6^T along with the purple non-sulfur strain ML42 (Yurkova et al., 2002) define a novel lineage. As bootstrap values are at this level very low, the branching point may change when novel sequences will be included. The phylogenetic distance between ML6^T and ML42 (96.9%) is that generally found for well separated species. However, physiological differences clearly place them in different genera (Yurkova et al., 2002). Strain ML6^T shares less than 93.0% 16S rDNA gene sequence similarity with its phylogenetically closest described relatives, the

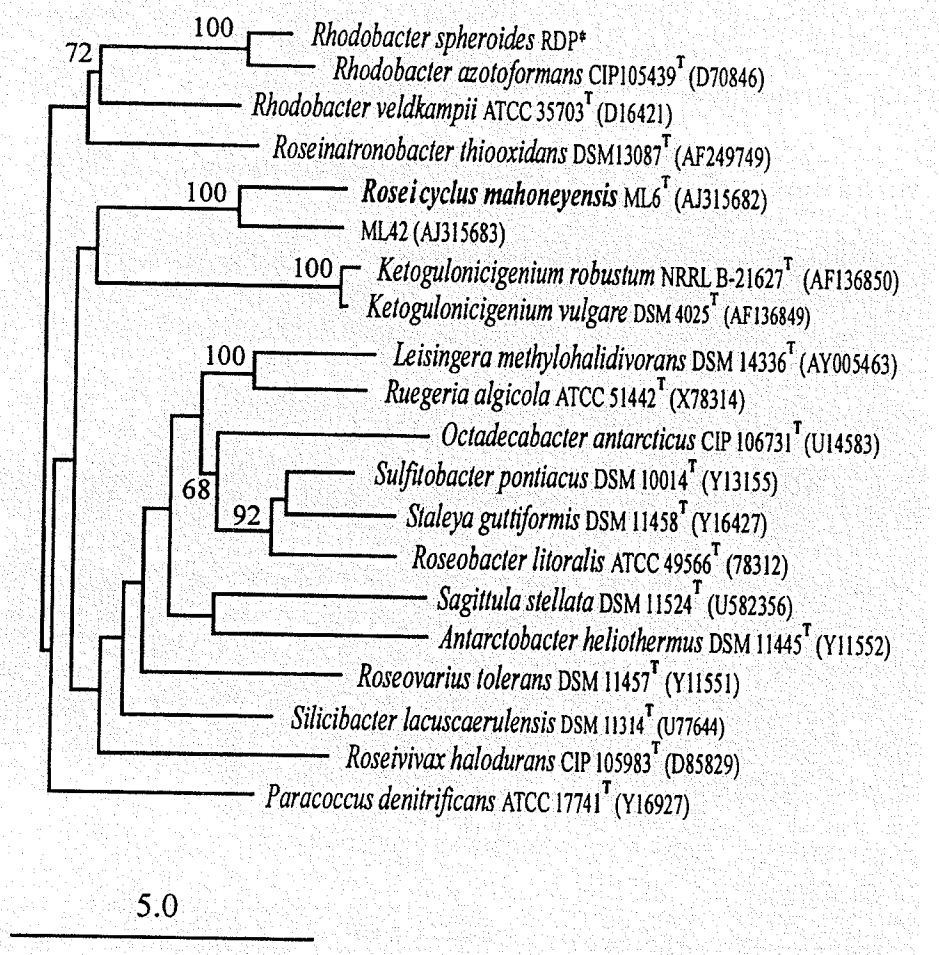

Fig. 4.3. Neighbour-joining dendrogram of 16S rDNA relatedness showing the position of *Roseicyclus mahoneyensis* strain ML6^T and its phylogenetic neighbours, i.e., the purple non-sulfur strain ML42, members of the genera *Ketogulonicigenium* and other members of the *Roseobacter* clade, α-3-cluster of the *Proteobacteria*. Bootstrap values (500 re-samplings) that support branching points above 90% confidence are indicated. The scale bar represents 5 nucleotide substitutions per 100 sequence positions. The tree was rooted with the 16S rRNA gene sequences of other members of the subclass α-*Proteobacteria*.

*, sequence not deposited in EMBL but in the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/html).

non-phototrophic genera *Octadecabacter* (92.0-92.9%) and *Ketogulonicigenium* (92.2-92.6%) and the aerobic phototrophic species *Roseivivax halodurans* (92.2-92.9%) and *Roseovarius tolerans* (91.7-92.4%).

4.5. Concluding remarks.

The presence of BChl *a* incorporated into RC, LH I and LH II complexes, the inability to grow photoheterotrophically under anaerobic conditions, the strong inhibition of BChl synthesis by light, and the absence of an ICM system prompt us to conclude that all strains described in this work are indeed members of the APB.

The phylogenetic analysis reveals that these strains have identical rDNA sequences, and form a distinct branch closely related to the chemotrophic genera *Octadecabacter* and *Ketogulonicigenium*, as well as to phototrophs of the genera *Roseivivax* and *Roseovarius*.

Morphological, physiological and biochemical properties allow us to easily differentiate new strains from their close phylogenetic neighbors *Octadecabacter* and *Ketogulonicigenium*. Members of *Octadecabacter* are obligate psychrophiles and form rod shaped cells containing gas vacuoles (Gosink et al., 1997), whereas *Ketogulonicigenium* sp. are facultatively anaerobic, ovoid rods that exhibit a relatively narrow pH, temperature and salinity range (Urbance et al., 2001). Neither *Octadecabacter* nor *Ketogulonigenium* form vibrioid or cyclical cells characteristic of our Mahoney Lake isolates, and neither contain BChl *a* or carotenoid pigments, although all species of *Ketogulonicigenium* produce an unidentified water soluble brown pigment.

Additionally, the strains represented by $ML6^T$ differ significantly from their closest phototrophic relatives, *Roseivivax* and *Roseovarius*, in terms of their cellular morphology and photosynthetic light harvesting apparatus. Although both were isolated from a similar habitat (i.e. a saline lake), and exhibit broad tolerance to saline conditions similar to that found in Mahoney Lake isolates, they do not produce a peripheral LH II complex and form regular motile rods (Suzuki et al., 1999; Labrenz et al., 1999).

Based on these important taxonomical markers, and low 16S rDNA sequence similarity (<93.0%) with closest phylogenetic relatives, we therefore propose the new genus *Roseicyclus*, with the type species *R. mahoneyensis*.

4.6. Description of *Roseicyclus* gen. nov.

Roseicyclus (Ro.se.i.cyc'lus. M.L. adj. *roseus*, rose, pink; M.L. n. *cyclus*, cycle; M.L. masc. n. *Roseicyclus*, pink cyclic bacterium).

Cells are Gram negative, ovoid to elongated rods, vibrioid or almost cyclical. Produce pointed enlarged areas of the periplasm located at the poles. Non-motile, divide by symmetric or asymmetric constrictions. Cultures are pink-purple to purple due to production of carotenoids and BChl *a*. Produce both LH I complex with an absorption peak at 870-1 nm, and an interesting LH II complex with one absorption peak at 805-6 nm. No growth occurs anaerobically in the light. Obligately aerobic, no fermentation or dissimilatory denitrification observed.

The habitat is a saline, Na_2SO_4-dominated lake. Member of the α-subclass of the *Proteobacteria*. The type species is *Roseicyclus mahoneyensis*.

4.7. Description of *R. mahoneyensis* sp. nov.

R. mahoneyensis (ma.ho.ney.en'sis. N.L. masc. adj. *mahoneyensis* from Mahoney Lake, where the species was originally isolated).

Gram negative pink-purple to purple elongated rods (0.6x2.6 μm) to vibrioid cells (0.6-0.7x2.7-3.0 μm). Non-motile.

Cells contain BChl *a* and carotenoid pigments. BChl gives *in vivo* absorption spectrum peaks at 805 to 806 and 870 to 871 nm. Aerobic organoheterotroph and facultative photoheterotroph. Best substrate for growth is yeast extract; growth also occurs on acetate, pyruvate, glutamate, butyrate, citrate, malate, succinate, lactate, fructose and glucose, depending on the strain. Strains differ in their ability to hydrolyze starch and Tween 60, all strains hydrolyze gelatin.

Optimum temperature for growth is 30°C with growth occurring as low as 4°C and as high as 37°C, depending on the strain. Absolute requirement for saline conditions with growth occurring over a wide range of NaCl and Na_2SO_4 concentrations from 0.5 to 10%. Growth occurs over a wide range of pH values from 6.0 to 11.0. May or may not be resistant to a variety of antibiotics including: penicillin G, streptomycin, tetracycline, ampicillin, kanamycin and nalidixic acid, dependant on the strain. Requires an as yet unidentified growth factor present in yeast extract.

The habitat is the meromictic saline Mahoney Lake in south central British Columbia, Canada. DNA base composition is 66.2 mol% G+C. The type strain is $ML6^T$ ($DSMZ=16097^T$, VKM=B-2346)

4.8. Acknowledgements.

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Chapter 5.

Porphyrobacter meromictius sp. nov., an appendaged bacterium, that produces bacteriochlorophyll *a*.

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(To be submitted in taxonomic note format)

The material presented in Chapter 5 is derived primarily from experiments completed by Christopher Rathgeber. Coauthors contributing to this paper were Natalia Yurkova, who began the characterization of the strains described herein; Erko Stackebrandt, who performed the phylogenetic analysis; Peter Schumann, who performed the G+C determinations; Elaine Humphrey, who prepared thin sections and assisted with electron microscopy and Vladimir Yurkov, who guided the project. This research was initiated in the lab of J. Thomas Beatty.

5.1. Abstract.

Four Gram-negative strains (ML4^T, ML19, ML31, ML32) of non-motile, appendaged, budding bacteria were isolated from the meromictic Mahoney Lake in British Columbia, Canada. The strains were red to brown-red in color and produced bacteriochlorophyll *a* incorporated into photosynthetic pigment-protein complexes. Phylogenetic analysis has placed these strains within the class *Alphaproteobacteria*, with the closest relatives being members of the genera *Erythrobacter*, *Porphyrobacter* and *Erythromicrobium*. Morphological features warrant their inclusion within the genus *Porphyrobacter* and these strains can be readily distinguished from other species of this genus on the basis of a mesophilic temperature range, a broad pH range, tolerance to extremely high NaCl and Na_2SO_4 concentrations, in keeping with the environment from which they were isolated, a Na_2SO_4-dominated meromictic lake. These isolates utilize a variety of organic substrates for aerobic chemoheterotrophic growth, and do not grow under anaerobic conditions, either in the presence or absence of light. All strains require vitamin B_{12}, and strains ML4^T and ML19 require biotin. The DNA G + C contents ranged from 62.2 – 64.9 mol%. Phenotypic and phyletic data support the classification of strains ML4^T, ML19, ML31 and ML32 as a novel *Porphyrobacter* species for which the name *Porphyrobacter meromictius* sp. nov. is proposed.

5.2. Introduction.

The APB are distinguished from other bacteria by the presence of BChl *a* incorporated into photosynthetic units, and the inability to use these photosynthetic units for phototrophic growth under anaerobic conditions (Yurkov and Beatty, 1998;

Rathgeber et al., 2004). APB fall primarily into the class *Alphaproteobacteria*, and are intermixed with both phototrophic and non-phototrophic genera. This has led to some confusion with regards to the taxonomy of this heterogeneous class (Yurkov and Beatty, 1998; Yurkov and Csotonyi, 2003), in which non-phototrophic representatives have sometimes been classified as species within the existing phototrophic genera based primarily on phylogenetic distance, despite a lack of phenotypic similarities, *Erythrobacter citreus* for example (Denner et al., 2002). Conversely, in other cases classical taxonomic markers have been given precedence and closely related strains have been classified into novel non-phototrophic genera, as for example the not yet validated taxon '*Lutibacterium anuloederans*' (Chung and King, 2001).

The first described genus of APB, *Erythrobacter*, was originally defined for long slender rod shaped, aerobic chemoorganotrophs that produce BChl *a* and carotenoids, and reproduce by binary fission (Shiba and Simidu, 1982). The genus *Porphyrobacter* was designated in 1993 to include BChl containing aerobes, branching with *Erythrobacter longus* within the *Alphaproteobacteria*. *Porphyrobacter* differed from *Erythrobacter* in habitat, originating from a freshwater environment, and by an ultrastructural resemblance to the *Planctomycetales*, a tendency toward pleomorphism, replication by polar growth or budding and the production of multifibrillar stalk-like structures, as well as the absence of cyt *c* oxidase and a lack of vitamin requirements (Fuerst et al., 1993). This genus now includes five species, the type species *Porphyrobacter neustonesis,* the moderate thermophiles *Porphyrobacter tepidarius* (Hanada et al., 1997) and *Porphyrobacter cryptus* (Rainey et al., 2003), the budding *Porphyrobacter sanguineus* (Hiraishi et al., 2002) and the marine microorganism *Porphyrobacter donghaensis* (Yoon et al., 2004b).

In this study we report the characterization of four halotolerant reddish-brown, BChl-containing strains that form appendages and reproduce by budding as a novel species within the genus *Porphyrobacter*.

5.3. Methods, results and discussion (format of taxonomic note).

Strains ML19, ML31 and ML32 were isolated from the surface, and strain ML4^T from 3 m depth, of the Na_2SO_4 dominated meromictic Mahoney Lake in the Okanagan Valley of British Columbia, Canada (Yurkova et al., 2002). Medium N1 (ML4^T, ML19) and medium N4 (ML31, ML32) or modifications thereof were used for all experiments unless otherwise noted (Yurkova et al., 2002). Strains could be stored on agar media at 4°C for several months. For long term storage a thick cell suspension was supplemented with 30% glycerol and frozen at -70°C. Morphology and cytology were examined by phase contrast microscopy (Zeiss Axioskop 2 microscope) and transmission electron microscopy as described (Yurkova et al. 2002). The ability to grow at different salinities was tested with concentrations of NaCl or Na_2SO_4 from 0 to 14% (Rathgeber et al., 2005). Temperature and pH range, utilization of soluble organic substrates, ability to ferment sugars and reduce nitrate, catalase and oxidase production, and antibiotic sensitivity were determined (Yurkov and Van Gemerden, 1993; Yurkov et al., 1994). Phototrophic growth under anaerobic conditions was tested in the above media, in basal media designed for purple sulfur bacteria containing H_2S or $Na_2S_2O_3$ and CO_2 with or without acetate, and in a basal medium for purple non-sulfur bacteria containing acetate, malate or succinate as the sole source of organic carbon (Imhoff, 1988; Rathgeber et al., 2005). Spectral absorption measurements of whole cells grown under aerobic conditions

in liquid culture were recorded using a Hitachi U-2010 spectrophotometer (Rathgeber et al., 2005).

DNA G + C content was determined using HPLC (Shimadzu) as described (Tamaoka and Komagata, 1984) of nucleotides obtained according to Mesbah and Whitman (1989). Extraction of genomic DNA, PCR-mediated amplification of 16S rRNA gene sequences and direct sequencing of PCR products were carried out as in Rainey *et al.* (1996). Sequence reaction mixtures were electrophoresed using a model 373A automatic DNA sequencer (Applied Biosystems). The 16S sequences were aligned with published sequences obtained from the EMBL nucleotide sequence database and the Ribosomal Database Project, using the ae2 editor (Maidak et al., 1996), and similarity values were determined.

Strains ML4^T, ML19, ML31 and ML32 formed red to reddish-brown colonies on agar plates dependent on age, and appear red-brown in liquid culture. Absorption spectra of whole cells, grown aerobically in the dark, revealed that this pigmentation was due to the presence of BChl *a* incorporated into RC and LH I complexes, evidenced by peaks at 806-808 and 866-867 nm respectively, and carotenoid pigments giving rise to peaks at 466-468 and 489-491 nm (Fig. 5.1). This type of photosynthetic apparatus is common to most *Porphyrobacter* species (Fuerst et al., 1993; Hanada, et al., 1997; Rainey et al., 2003; Yoon et al., 2004b). However, *P. sanguineus*, the sole exception, displays an absorption maxima at 814 nm that may represent an additional LH II component (Hiraishi et al., 2002), although the cause of this absorption maximum remains unclear,

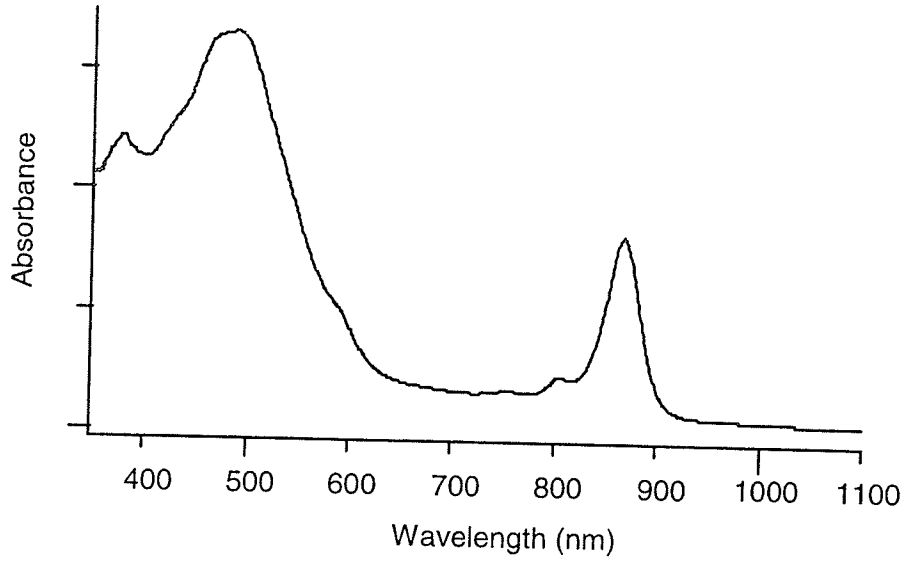

Fig. 5.1. Absorption spectra of strain ML4^T whole cells grown aerobically in the dark show a peak at 806 nm, indicative of a photosynthetic reaction center, and at 867 nm, indicative of a light harvesting I complex.

as such a blue-shifted LH II has not previously been observed in APB. Cells grown aerobically in the presence of continuous illumination lack or have greatly reduced absorption peaks in both the 806 and 867 nm regions indicating that production of the photosynthetic apparatus is strongly inhibited by light. All of the isolates were incapable of anaerobic photosynthetic growth in all media tested, confirming that these isolates are members of the APB.

Morphology was examined in exponential phase cells grown in liquid medium under aerobic conditions. Each of the strains formed short rods (0.5 × 1.5 μm) to ovoid shaped cells (0.5 × 1.0 μm) and reproduced by budding or constrictions. Motility was not observed in either liquid cultures or cultures grown on agar plates, and flagella were not observed in electron microscopy. There was a tendency to form chains of about six cells, although longer chains were observed in strain ML19.

Transmission electron microscopy of strains ML4^T and ML19 (both thin sections and negative stains) revealed many as yet unexplained structures (Yurkova et al., 2002). Negatively stained cells of ML19 showed an unusual type of connective material between cells that appeared to consist of a bubble-like formation with a tubular structure located at the center (Fig. 5.2a). This type of connective material has not been observed before in the domain *Bacteria*. Strain ML19 also produced an appendage (Fig. 5.2b) similar to that seen in *P. neustonensis* (Fuerst et al., 1993). The presence of these structures was confirmed in thin sections (Fig. 5.2c).

Strain ML4^T excreted large amounts of capsular material, causing aggregation among cells, and also produced an extracellular protrusion, although this structure resembled a chain of beads (Fig. 5.2d, e) (Yurkova et al., 2002) that was clearly different

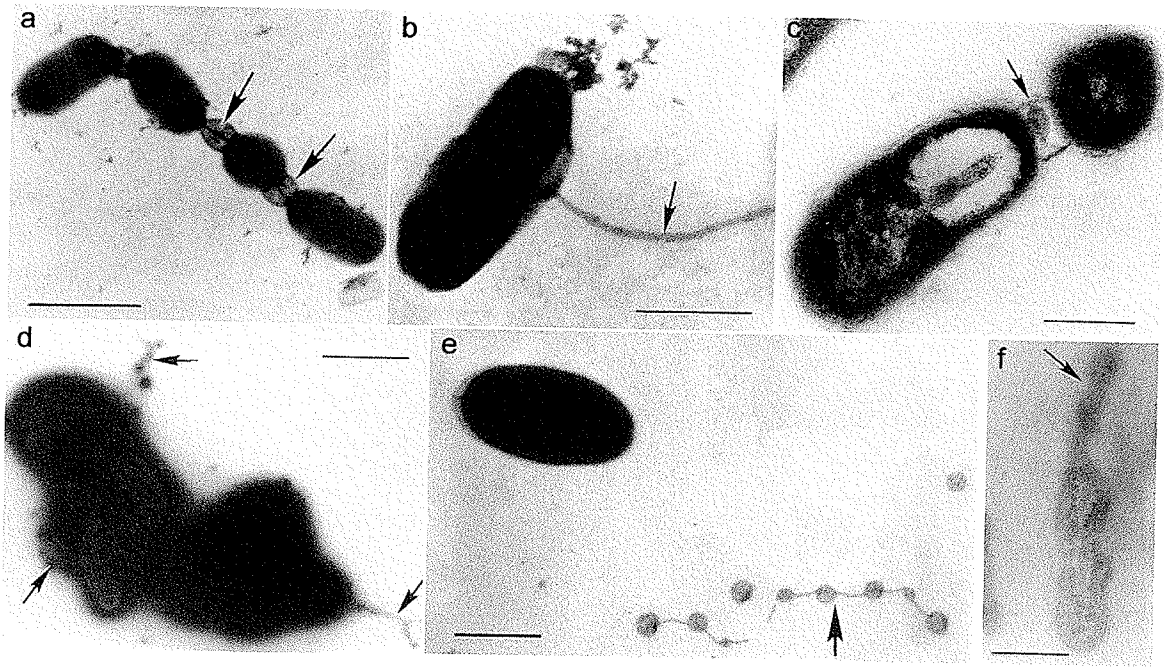

Fig. 5.2. Negatively stained micrographs of strain ML19 reveal (a) unusual connective material and (b) a stalk-like structure. (c) The presence of these structures was confirmed in thin sections. Strain ML4^T negative stains show (d) an unusual extracellular protrusion and (e) an appendage detached from the cell. Thin sections (f) show a tendency toward pleomorphism and prostheca formation. Bars: (a,d) 1.0 μm, (b,f) 0.5 μm, (c,e) 0.25 μm.

from the stalk of *P. neustonesis*. Thin sections of ML4^T revealed cells with a tendency toward pleomorphism and possible production of a prostheca-like structure (Fig. 5.2f).

The Gram-negative cell wall structure was confirmed in thin sections, and as expected for APB, ICM formations, of the type usually found in AnAP, were not observed (Fig. 5.2c, f).

Strains ML4^T, ML19, ML31 and ML32 were similar in terms of physiological properties. They tolerated a wide range of pH, with growth occurring between pH 5.5 and 10.0, but not at pH 5.0 or 11.0. Saline conditions were not required, and all strains grew well in the absence of added NaCl or Na_2SO_4. Strains ML4^T, ML19 and ML32 tolerated up to 8% NaCl, whereas ML31 grew at up to 10% NaCl. Each of the strains exhibited slightly higher tolerance to Na_2SO_4, the dominant salt at the site of isolation (Northcote and Halsey, 1969), with ML19 tolerating 10%, ML4^T and ML32 tolerating 13%, and ML31 withstanding 14% Na_2SO_4, the highest concentration tested . In all cases however, optimal growth was achieved at concentrations between 0-2% of the respective salt. The temperature range was mesophilic, with growth occurring between 10 and 37 °C, but not at 5 or 45 °C.

Catalase and oxidase were produced by all of the strains, as well as extracellular lipases capable of hydrolyzing Tween 60. Additionally ML4^T, ML19 and ML31 produced extracellular proteinases capable of hydrolyzing gelatin, and ML19 and ML32 produced extracellular amylases.

These strains appear to be nutritionally versatile, able to use a variety of simple organic compounds as the sole source of carbon and energy for aerobic chemoorganotrophic growth. All strains use acetate, pyruvate, glutamate, butyrate,

malate, succinate and lactate. Additionally strain ML4^T can use glucose, and strains ML31 and ML32 can use glucose, fructose, citrate and formate, and even appear capable of very weak growth on ethanol and methanol. The strains did not ferment glucose or fructose, and were not capable of dissimilative nitrate reduction under anaerobic conditions. All of the strains require the addition of vitamin B_{12} to the growth medium, and strains ML4^T and ML19 have a requirement for biotin. The addition of biotin stimulates the growth of ML31 and ML32, although they are capable of growth in its absence.

The strains were all susceptible to chloramphenicol (30 μg), tetracycline (30 μg) and kanamycin (30 μg), and ML31 and ML32 were susceptible to penicillin G (10 units). Strain ML31 alone was susceptible to ampicillin (2 μg). All were able to grow in the presence of streptomycin (10 μg), polymixin B (50 μg) and nalidixic acid (30 μg).

In a previous study partial 16S rRNA gene sequences (>400 nucleotides) identified these and other APB isolates from Mahoney Lake as members of the class *Alphaproteobacteria*, and revealed that the partial 16S rRNA genes of ML4^T, ML19, ML31 and ML32 were nearly identical to each other, and most closely related (99.7-98.8%) to an undescribed strain of *Porphyrobacter* (Yurkova et al., 2002). Based on these data, and on biochemical properties, ML4^T, ML19 and ML31 were chosen for nearly complete 16S rRNA gene sequencing (>1430 nucleotides), which revealed that the 16S rRNA genes of ML4^T and ML19 are identical and differ only slightly from that of ML31 (99.8%). Phylogenetic analysis based on 16S rRNA gene sequences placed these strains within the phyletic cluster of *Erythrobacter-Porphyrobacter-Erythromicrobium* (Fig. 5.3). The nearest neighbors are the aerobic phototrophic species *Erythrobacter*

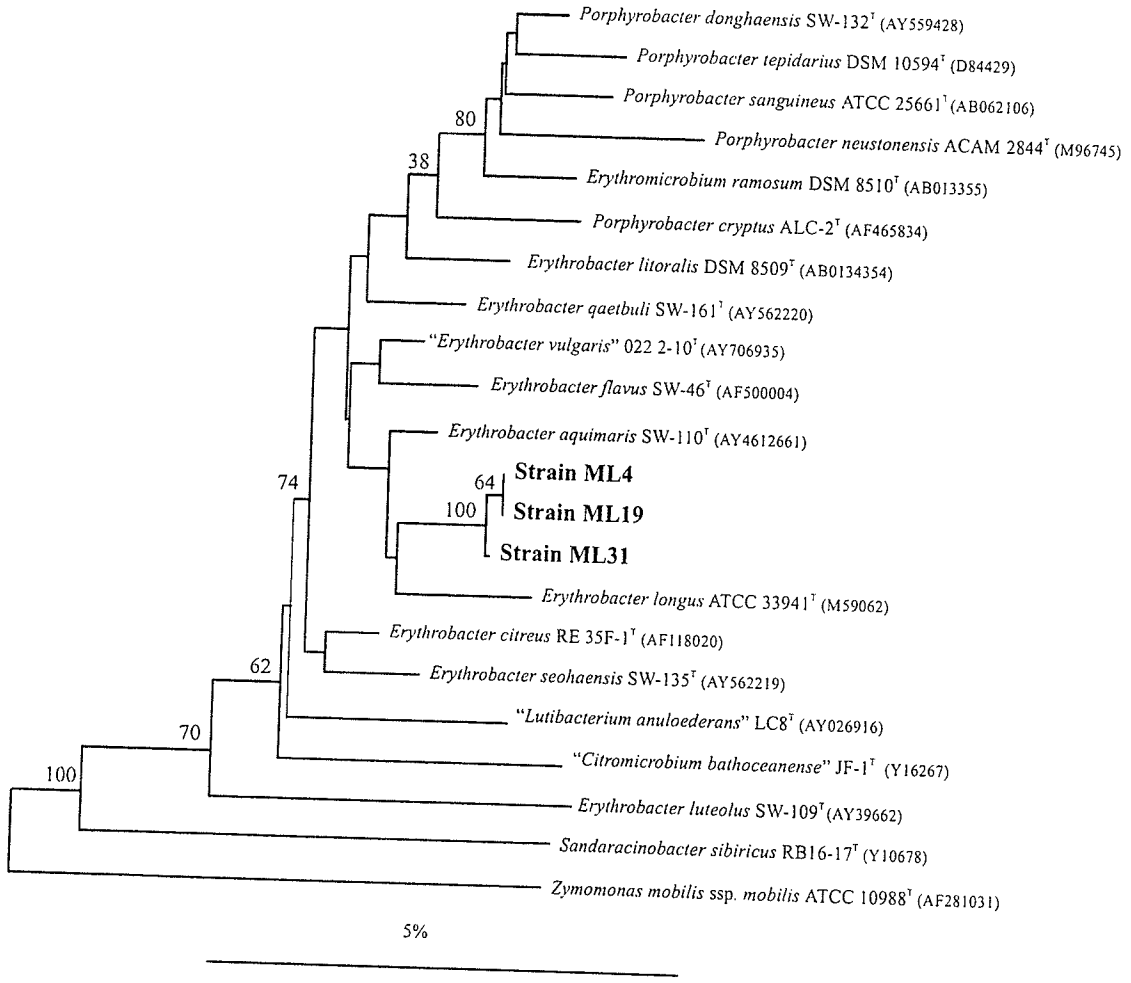

Fig. 5.3. Neighbor-joining dendrogram of 16S rRNA gene sequence relatedness shows that strains ML4^T, ML19 and ML31 form a monophyletic group within the *Alphaproteobacteria* supported by 100% bootstrap values (500 resamplings). The position of these sequences relative to the existing genera is unclear due to relatively low confidence for branching points within the α-4 cluster. *Zymomonas mobilis* ssp. *mobilis* was used as an outgroup. Bar, 5 substitutions per 100 sequence positions.

longus and *Erythrobacter litoralis* (97.4-97.7%) and the non-phototrophic species recently ascribed to the genus *Erythrobacter* (92.6-98.1%), as well as phototrophs of the genera *Porphyrobacter* (94.5-96.7%) and *Erythromicrobium* (95.8-96.1%). The G + C contents of the DNA of strains ML4^T, ML19 and ML31 were found to be 64.9, 62.2 and 63.6 mol%, respectively.

Assessing the taxonomic status of new strains related to the *Erythrobacter-Porphyrobacter-Erythromicrobium* cluster within the *Alphaproteobacteria* is complicated by relatively unclear phylogenetic tree topology. Regardless of whether the algorithm of De Soete (1983) or the neighbor joining or maximum parsimony algorithms (Felsenstein, 1993) were applied to the sequence alignment, branches separating these genera are generally not well supported by the bootstrap values (Rainey et al., 2003; this study). Our strains could be assigned to any of the three genera *Erythrobacter, Porphyrobacter* or *Erythromicrobium* based solely on phylogenetic distance. However, examination of classical taxonomic markers makes it apparent that these strains tend to fall within the taxonomic criteria originally described for the genus *Porphyrobacter* (Table 5.1). The production of BChl incorporated into a RC and LH I, but not LH II (as is the case for *Erythromicrobium*, which contains an unusual blue-shifted LH II), a tendency toward pleomorphism, production of appendages and reproduction by budding. Whereas neither members of *Erythrobacter* nor members of *Erythromicrobium* form appendages or reproduce by budding, and neither are pleomorphic, forming relatively long rod shaped cells and very long thread-like cells respectively (Yurkov and Beatty, 1998). These

Table 5.1. Comparative phenotypic properties between *Porphyrobacter meromictius* and closely related species of the *Alphaproteobacteria.*

Property	Strain				
	1	2	3	4	5
Habitat	Meromictic lake	Freshwater lake	Brackish-Marine	Hotspring	Brackish hotspring
Color	Red-brown	Orange-red	Orange-red	Red-orange	Orange
BChl *a*	+	+	+	+	+
LH II	-	-	+	-	-
Stalk	+	+	-	-	-
Budding	+	+	+	na	-
Motility	-	+	+	+	-
Growth at					
10 °C	+	+	-	-	-
45 °C	-	-	+	+	+
8% NaCl	+	-	na	na	-
Hydrolysis of					
Gelatin	V(+)	-	-	+	-
Starch	V(-)	-	-	+	+
Required or stimulates					
Biotin	V*(+)	-	na	na	+
Vitamin B_{12}	+	-	na	na	-
Mol% G + C	62.2-64.9	65.7-66.4	63.8-64.0	66.2	65.0

Taxa: 1, *P. meromictius*; 2, *P. neustonensis* (Fuerst et al., 1993); 3, *P. sanguineus* (Hiraishi et al., 2002); 4, *P. cryptus* (Rainey et al., 2003); 5, *P. tepidarius* (Hanada et al., 1997); 6, *P. donghaensis* (Yoon et al., 2004b); 7, *E. aquimaris* (Yoon et al., 2004a); 8, *E. longus* (Shiba and Simidu, 1982); 9, *E. ramosum* (Yurkov et al., 1994).
Symbols: +, growth occurs, substrate is hydrolyzed or vitamin is required; -, growth does not occur, substrate is not hydrolyzed or vitamin is not required; V, results vary between strains; na, data not available; LH II, light harvesting II complex. Data in parentheses are for the type strain.
* Biotin stimulates growth for strains ML31 and ML32, whereas strains $ML4^T$ and ML19 require biotin.

Table 5.1. Comparative phenotypic properties between *Porphyrobacter meromictius* and closely related species of the *Alphaproteobacteria* (Continued).

Property	Species			
	6	7	8	9
Habitat	Marine	Marine	Marine	Alkaline spring
Color	Red-orange	Orange	Orange	Orange
BChl *a*	+	-	+	+
LH II	-	-	-	+
Stalk	-	-	-	-
Budding	na	na	-	-
Motility	+	-	+	+
Growth at				
10 °C	+	+	na	na
45 °C	+	-	na	na
8 % NaCl	-	+	-	-
Hydrolysis of				
Gelatin	-	-	+	-
Starch	+	V(+)	-	-
Required or stimulates				
Biotin	na	na	+	na
Vitamin B_{12}	na	na	-	na
Mol% G + C	65.9-66.8	62.2-62.9	60-64	63.6-64.2

Taxa: 1, *P. meromictius*; 2, *P. neustonensis* (Fuerst et al., 1993); 3, *P. sanguineus* (Hiraishi et al., 2002); 4, *P. cryptus* (Rainey et al., 2003); 5, *P. tepidarius* (Hanada et al., 1997); 6, *P. donghaensis* (Yoon et al., 2004b); 7, *E. aquimaris* (Yoon et al., 2004a); 8, *E. longus* (Shiba and Simidu, 1982); 9, *E. ramosum* (Yurkov et al., 1994).
Symbols: +, growth occurs, substrate is hydrolyzed or vitamin is required; -, growth does not occur, substrate is not hydrolyzed or vitamin is not required; V, results vary between strains; na, data not available; LH II, light harvesting II complex. Data in parentheses are for the type strain.
* Biotin stimulates growth for strains ML31 and ML32, whereas strains $ML4^T$ and ML19 require biotin.

significant morphological features strongly differentiate the genus *Porphyrobacter* from either *Erythrobacter* or *Erythromicrobium*.

Porphyrobacter was originally proposed to include APB of freshwater origin, however the majority of species since added to the genus originate from marine or brackish environments. Because we feel that the location of origin should remain an important taxonomic marker, the strains from Mahoney Lake present an interesting quandary. Although the Mahoney Lake monomolimnion may have salinity as high as 40‰ (Hall and Northcote, 1986), the salinity of surface waters on the day of sampling was only 5‰ (Yurkova et al., 2002) which is typical of freshwater systems. As all of our strains are capable of robust growth in a medium devoid of added NaCl or Na_2SO_4, it is appropriate to include these strains within the genus *Porphyrobacter*.

Although our isolates fit well within the specifics of the genus *Porphyrobacter*, several important properties clearly differentiate them from other known species (Table 5.1). The ability to grow at very high salinities, and over a broad range of pH values are likely essential properties for survival in their natural environment. As well phylogenetic analysis indicates that strains ML4^T, ML19 and ML31 form a distinct cluster clearly separate from other species within the *Alphaproteobacteria*.

5.4. Description of *Porphyrobacter meromictius* sp. nov.

Porphyrobacter meromictius (me.ro.mic'ti.us. Gr. neut. n. *meros* part, L. adj. part *mictus* mixed, L. adj. suffix *–ius* belonging to, N.L. masc. adj. *meromictius* belonging to meromictic environments, lakes).

Cultures are pigmented red to red-brown due to production of BChl *a* giving rise to absorption peaks at 806-808 and 866-867 nm, and carotenoids with peaks at 466-468 and 489-491 nm. Cells are short rods (0.5 × 1.0 to 0.5 × 1.5 μm) or pleomorphic, may occur in chains of six cells or more, and reproduce by budding or constrictions. May form appendages or other extracellular protrusions. Obligate aerobic chemoorganoheterotroph and facultative photoheterotroph. Growth occurs on acetate, pyruvate, glutamate, butyrate, malate, succinate and lactate; some strains also use citrate, formate, fructose and glucose. Strains differ in their ability to hydrolyze starch and gelatin; all strains hydrolyze Tween 60. Exhibits a mesophilic temperature range between 10-37°C. Tolerates wide range of salinity from 0 to 8-10% NaCl or 0 to 10-14% Na_2SO_4; optimum salinity is 0-2% NaCl or Na_2SO_4. Growth occurs between pH 5.5 and 10.0. Requires vitamin B_{12}, biotin is either required or stimulates growth depending on the strain. Catalase and oxidase positive. Sensitive to chloramphenicol, tetracycline, kanamycin; resistant to streptomycin, polymixin B, nalidixic acid; some strains may be resistant to penicillin G and ampicillin. The DNA G + C content is 62.2-64.9 mol%.

The habitat is a Na_2SO_4-dominated meromictic lake, in south-central British Columbia, Canada. The type strain is ML4^T (= DSM 18336^T = VKM B-2405^T).

5.5. Acknowledgements .

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Chapter 6.

Metalloid reducing bacteria isolated from deep ocean hydrothermal vents of the Juan de Fuca Ridge, *Pseudoalteromonas telluritireducens* sp. nov. and *Pseudoalteromonas spiralis* sp. nov.

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The material presented in Chapter 6 is derived primarily from experiments completed by Christopher Rathgeber. Coauthors contributing to this paper were Natalia Yurkova, who began the characterization of the strains described herein; Erko Stackebrandt, who performed the phylogenetic analysis; Peter Schumann, who performed the G+C determinations and riboprinting; Elaine Humphrey, who prepared thin sections and assisted with electron microscopy and Vladimir Yurkov, who guided the project. This research was initiated in the lab of J. Thomas Beatty.

6.1. Abstract.

Five strains of Gram negative, rod, curved rod and spiral shaped bacteria were isolated from the vicinity of deep ocean hydrothermal vents along the Main Endeavour Segment of the Juan de Fuca Ridge in the Pacific Ocean. All strains showed remarkable resistance to high levels of toxic metalloid oxyanions, and were capable of reducing the oxyanions tellurite and selenite to their less toxic elemental forms. Phylogenetic analysis of four strains identified these isolates as close relatives of the genus *Pseudoalteromonas* within the class *Gammaproteobacteria*. *Pseudoalteromonas agarivorans* was the closest relative of strains Te-1-1 and Se-1-2-red^T, with respectively 99.5 and 99.8% 16S rDNA sequence similarity. Strain Te-2-2^T was most closely related to *Pseudoalteromonas paragorgicola*, with 99.8% 16S rDNA sequence similarity. The DNA G+C base composition was 39.6 to 41.8 mol%, in agreement with other members of the genus *Pseudoalteromonas*. However the isolates showed important morphological and physiological differences from previously described species of this genus, with one group forming rod shaped bacteria typical of *Pseudoalteromonas* and the other forming vibrioid to spiral shaped cells. Based on these differences, and on phylogenetic data, we propose the creation of the new species *Pseudoalteromonas telluritireducens* sp. nov., with strain Se-1-2-red^T (DSMZ=16098^T = VKM B-2382^T) as the type strain, and *Pseudoalteromonas spiralis* sp. nov., with strain Te-2-2^T (DSMZ=16099^T = VKM B-2383^T) as the type strain.

6.2. Introduction.

Tellurite and selenite are toxic metalloid oxyanions, which can be found in low amounts in natural environments, and in higher concentrations in soil and water polluted by industrial processes (Avazeri et al., 1997). Toxicity of these ions has been attributed to their strong oxidizing activity, which may interfere with basic cellular processes (Summers and Jacoby, 1977).

Intrinsic low level resistance to tellurite (TeO_3^{-2}) has been reported for some Gram positive bacteria, although the mechanism for this resistance is not clearly understood. In Gram negative bacteria, plasmid mediated resistance, as well as resistance resulting from a secondary function of nitrate reductase, has been demonstrated (Summers and Jacoby, 1977; Walter and Taylor, 1989; Avazeri et al., 1997). Reduction of tellurite results in black colonies on the surface of agar, and a black culture in liquid media due to the accumulation of intracellular deposits of elemental tellurium (Moore and Kaplan, 1992; Taylor et al., 1988).

Se, which occurs in nature primarily in the toxic oxidized forms selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), is a biologically essential element at low concentrations, but can also become toxic at higher levels. Bacterial reduction of selenite into non-toxic elemental Se has been suggested as one mechanism used to neutralize this toxic anion. Reduction of selenite results in dark orange to red colonies on agar, and dark orange to red culture in liquid media due to accumulations of the red amorphous elemental Se (Moore and Kaplan, 1992).

Chemical detoxification of metalloid polluted sites is expensive and can result in secondary adverse environmental effects, therefore the development of bioremediation is

an area of research with potentially useful applications. Several strains of bacteria, such as members of the purple non-sulfur bacterial family the *Rhodospirillaceae* (Moore and Kaplan, 1992; 1994; O'Gara et al., 1997), the aerobic phototrophic bacteria (Yurkov et al., 1996) and the hyperhalophillic *Archaea* (Pearion and Jablonski, 1999) have been reported to reduce high concentrations of metalloid oxyanions. Despite the discovery of these metalloid reducers, the development of an economical system for bioremediation of metal and metalloid contaminated sites is still in its infancy, and therefore the identification of new strains capable of reducing these compounds is of great importance.

Hydrothermal vents associated with the global oceanic ridge system support a vast, complex ecosystem on and beneath the deep ocean floor. Deep ocean vent fluids possess a wide range of chemical compositions, and often are enriched in metal sulfides, including iron, copper, calcium, silicon and zinc as well as metalloids (Feely et al., 1987; Butterfield et al., 1994). Thus this environment is an excellent site for the isolation of metalloid resistant microorganisms.

Previously we reported on the isolation of ten strains of metalloid reducing bacteria from the vicinity of deep ocean hydrothermal vent sites along the Main Endeavour Segment of the Juan de Fuca Ridge in the Pacific Ocean (Rathgeber et al., 2002), and described their resistance and ability to reduce extremely high levels of both tellurite and selenite.

In this paper we use phylogenetic analysis and physiological studies of five isolates to identify them as members of the marine, γ-proteobacterial genus *Pseudoalteromonas*. The genus *Pseudoalteromonas* was created in 1995 when 16S rDNA sequence analysis showed that although morphologically and physiologically similar,

significant difference existed between several species of the genus *Alteromonas* and the type species *Alteromonas macleodii* and was later emended to include species motile by polar, bipolar, or lateral flagella that decompose gelatin and Tween 80 (Ivanova et al., 2002).

The genus *Pseudoalteromonas* is comprised of Gram negative, rod and curved rod shaped bacteria. Most species are motile by means of a single unsheathed polar flagellum, although two species, *Pseudoalteromonas luteoviolacea*, and *Pseudoalteromonas denitrificans* (Enger et al., 1987; Gauthier et al., 1995), possess sheathed flagella. All members are strictly aerobic chemoorganotrophs, and although they are a physiologically heterogeneous group they all share the following traits: production of gelatinase, lipase, DNAse, lecithinase; utilization of D-glucose as sole source of carbon and energy; requirement of a seawater base for growth; and oxidase production, although catalase production is weak or irregular (Gauthier et al., 1995).

Although members of the genus *Pseudoalteromonas* and *Alteromonas* have been reported to produce extracellular polysaccharide materials with the ability to bind metals such as zinc, cadmium and lead (Loaec et al., 1998), no previously described member has been shown to resist or reduce high levels of tellurite and selenite. Based on phylogenetic data, differences in morphology and physiology, we herein propose the designation of two new species within the genus *Pseudoalteromonas*.

6.3. Methods.

6.3.1. Bacterial strains.

Two strains of vibrioid to spirillum shaped, metalloid reducing bacteria, Te-2-1 and Te-2-2^T were isolated from vent fluids below a hydrothermal vent flange. Three strains, Te-1-1, Te-1-2 and Se-1-2-red^T, consisting of rod shaped, slightly curved rod, as well as vibrioid and spirillum shaped cells, were isolated from samples of bacterial aggregates taken from Melarie Summit, all located along the Main Endeavour Segment of the Juan de Fuca Ridge (Rathgeber et al., 2002).

These strains were maintained and all experiments were performed on D/O medium (Rathgeber et al., 2002) unless otherwise stated. Long term storage of strains was achieved by freezing a dense suspension of cells, in D/O medium supplemented with 30% glycerol as a cryoprotectant, at -70° C.

The following *Pseudoalteromonas* spp. type strains were obtained from DSMZ and were maintained on Difco Marine Agar plates or RO NaCl plates (Yurkov et al., 1996): *P. agarivorans* DSM 14585^T; *P. atlantica* DSM 6839^T; *P. distincta* DSM 12749 ; *P. elyakovii* DSM 12747; *P. espejiana* DSM 9414^T; *P. paragorgicola* DSM 14403^T; *P. undina* DSM 6065^T.

6.3.2. Morphology and cytology.

The size and shape were determined from cells grown in unamended D/O, or in HM medium containing either 100 μg of K_2TeO_3 or 100 μg of Na_2SeO_3 per ml (Rathgeber et al., 2002).

Cytology was investigated using thin sections prepared by embedding bacteria in Epon after fixation with 2.5% glutaraldehyde and 1% osmium tetroxide as described (Kellenberger et al., 1958). Negative stains were performed by staining cells with 1.0% aqueous uranyl acetate.

6.3.3. Physiological and biochemical experiments.

Utilization of organic substrates, ability to grow at different pH values and different levels of salinity, catalase and oxidase production, and antibiotic sensitivity were determined as previously described (Yurkov and Van Gemerden, 1993; Yurkov et al., 1994). The ability to reduce nitrate and the ability to ferment sugars, as well as the ability to reduce metalloid oxyanions, both aerobically and anaerobically were determined previously (Rathgeber et al., 2002).

Resistance to and reduction of tellurite was examined in closely related *Pseudoalteromonas* spp. Strains were plated on HM (Rathgeber et al., 2002), RO NaCL (Yurkov et al., 1999) and Difco Marine Agar plates containing 0, 10, 100, 500, 1000 and 2000 µg per ml K_2TeO_3. These plates were incubated aerobically at 28 °C for 5 days, and were evaluated as described (Rathgeber et al., 2002).

6.3.4. G + C content determination and riboprinting.

The DNA base composition was determined by HPLC (Tamaoka and Komagata, 1984) of nucleotides obtained according to Mesbah and Whitman (1989). Ribotyping was carried out with the RiboPrinter microbial characterization system (Qualicon, DuPont),

using the restriction enzymes *Eco*RI and *Pvu*II , according to the instruction of the manufacturer.

6.3.5. Phylogenetic analysis.

Extraction of genomic DNA, PCR-mediated amplification of 16S rDNA and direct sequencing of the purified PCR product were done as described (Rainey et al., 1996). The 16S rDNA sequences (> 1430 bases) were aligned with published sequences obtained from the EMBL Nucleotide Sequence Database (Cambridge, UK) and the Ribosomal Database Project (RDP) using the ae2 editor (Maidak et al., 1996). Evolutionary distances were calculated by the method of Jukes and Cantor (1969). Phylogenetic dendrograms were constructed as described by DeSoete (1983). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 500 resamplings (Felsenstein, 1985).

6.4. Results and discussion.

6.4.1. Morphology and cytology.

Two strains (Te-1-1, Se-1-2-red^T) form ovoid to rod shaped cells seemingly dependant on age of the culture. The mature mother cell (0.8 to 1.0 × 2.4 to 2.8 μm) divides by binary fission to form two ovoid (sometimes almost coccoid: 0.7 to 0.9 × 1.4 to 1.9 μm) cells. These strains are motile, however flagellation has not been observed; they undergo interesting changes in morphology when grown in the presence of metalloid oxides. In the presence of selenite, rods become elongated, and develop light-refractive

Se globules. After about 72 hours most of these globules appear to be excreted from the cell. Cultures grown in the presence of tellurite however become more ovoid, and light-refracting Te globules tend to remain inside cells, even in aging cultures (Rathgeber et al., 2002).

The three remaining strains (Te-1-2, Te-2-1, Te-2-2^T) form vibrioid (0.6 to 0.8 × 1.9 to 2.4 μm) or spirillum (0.6 to 0.7 × 3.2 to 5.3 μm) shaped cells, seemingly dependent on age, with a predominance of long spirillum shaped cells found in older cultures. All strains in this group are highly motile possessing what appears to be a single polar sheathed flagellum (Fig. 6.1A), and produce extensive accumulations of extracellular material (Fig. 6.1B). When grown on metalloid oxide containing medium, these strains accumulate elemental metalloid globules inside the cytoplasm without a noticeable change in morphology (Rathgeber et al., 2002).

The interesting spirillum shape of strains Te-1-2, Te-2-1 and Te-2-2^T is clearly different from any species of the genus *Pseudoalteromonas* thus far described, which are reported to form straight or curved rods with length not greater than 3.0 μm (Gauthier et al., 1995).

All of the isolates stained Gram negative, and the presence of inner and outer membranes was confirmed in electron microscopic thin sections. The strains do not form spores, and no poly-β-hydroxybutyrate type storage granules were detected.

6.4.2. Diagnostic growth and physiological properties.

All of the strains are oxidase positive and produce catalase, demonstrated by vigorous bubbling of released O_2 when cells are exposed to H_2O_2. The genus

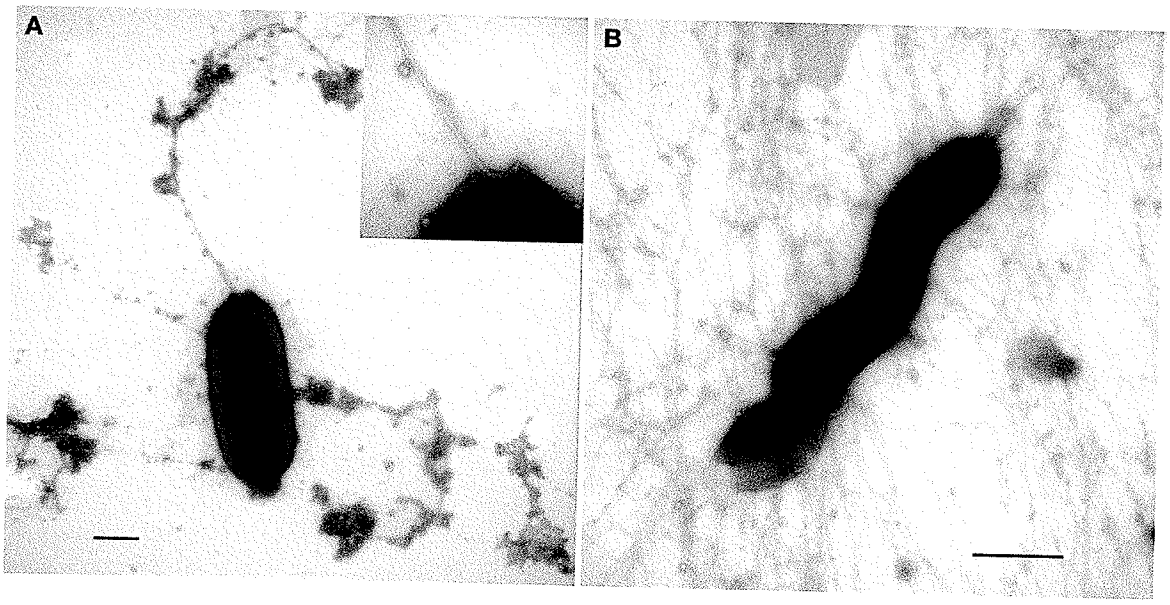

Fig. 6.1. Electron micrographs show (A) (A, inset) presence of a single polar sheathed flagellum in strain Te-2-1 and (B) extensive polysaccharide production by Te-1-2. Bars: 0.5 μm.

Pseudoalteromonas was initially described as having a weak or irregular catalase activity (Gauthier et al., 1995), however, recent additions to this genus have included both catalase positive and negative species (Bowman, 1998; Isnansetyo and Kamei, 2003; Romanenko et al., 2003).

Physiologically, the strains exhibit much variation, as has been shown in other members of the genus *Pseudoalteromonas* (Gauthier et al., 1995; Baumann et al., 1998), and no apparent trend can be discerned between the strains of either morphological group. Table 6.1 compares the physiological characteristics of the isolates.

All of our isolates were tolerant of a broad range of culture conditions, including salinity, temperature, pH and required saline medium for growth (0.5% NaCl), as is expected for this obligately marine genus, and grew in media containing up to 10% NaCl. Two exceptional strains of the rod shaped group, Se-1-3-red and Se-6-2-red, were capable of growth at concentrations of NaCl as high as 20%. In all strains optimal growth occurred between 1.5% and 8.0% NaCl. Halotolerance is not unusual for microorganisms isolated from the vicinity of deep ocean hydrotherms (Kaye and Baross, 2000), and has been reported in the species *Pseudoalteromonas prydzensis* (Bowman, 1998).

The ability to grow over a wide temperature range is unusual for members of *Pseudoalteromonas*, which typically are mesophiles. Some psychrophillic and psychrotrophic species have been identified, however the inability to grow at 37°C has previously been used as a distinguishing trait of the genus (Bowman, 1998). Thus the ability of all isolates to grow at 4°C and at 37°C and of two isolates (Te-1-1, Te-1-2) to grow at temperatures as high as 45°C further distances these strains from related species.

Table 6.1. Comparative physiological characteristics of the Juan de Fuca Ridge isolates.

Characteristic	Strain				
	Te-1-1	Te-1-2	Te-2-1	Te-2-2^T	Se-1-2-red^T
Growth at pH					
5.0	-	-	-	-	+
5.5	+	++	++	+	++
10.0	+	+	+	+	++
11.0	+	+	-	+	++
Growth at 45°	+	+	-	-	-
Utilization of*					
Acetate	+	+	+	+	-
Pyruvate	+	+	W	-	+
Glutamate	+	++	+	++	+
Butyrate	+	-	-	-	+
Citrate	+	-	-	+	+
Malate	-	-	-	+	+
Succinate	-	-	+	+	+
Lactate	-	-	+	+	+
Fructose	+	++	-	-	++
Glucose	+	+	+	++	+
Ethanol	-	-	-	-	++
Hydrolysis of					
Starch	+	+	-	-	+
Gelatin	+	-	-	-	+
Agar	++	-	-	-	++
Antibiotic Sensitivity†					
Penicillin G	+	-	-	-	-
Streptomycin	+	+	+	+	+
Tetracycline	+	+	-	-	+
Ampicillin	+	-	-	-	-
Kanamycin	+	+	+	+	+
Nalidixic acid	+	+	-	+	+

+, substrate is utilized, substrate is hydrolyzed or antibiotic sensitive; ++, substrate is utilized for very good growth; -, substrate is not utilized, substrate is not hydrolyzed or antibiotic resistance; W, weak growth; *, all strains grew very well on yeast extract and did not grow on methanol or formate; †, all strains were sensitive to chloramphenicol and polymixin B.

All strains hydrolyzed Tween 60 indicating the presence of lipolytic activity. Most strains hydrolyzed starch (amylolytic activity), but only 3 strains hydrolyzed gelatin (proteolytic activity) (Table 6.1). Gelatin hydrolysis is another characteristic that had been attributed to all members of the genus *Pseudoalteromonas* described thus far. Strains Te-1-1 and Se-1-2-red^T formed large clear halos on agar media which resulted in softening of the agar surface, indicating agarolytic activity.

The isolates were shown to utilize a wide variety of organic compounds as the sole source of carbon for aerobic heterotrophic growth. The best carbon sources for most of the strains were glutamate or glucose. Although anaerobic growth was not observed, strain Te-1-2 fermented fructose under microaerophilic conditions. Nitrate was not reduced by any of the strains. All strains were capable of growth in a vitamin free medium after repeated transfers, although strain Te-2-2^T showed slightly reduced growth in media lacking thiamine. This ability to utilize a wide range of organic carbon sources and the lack of required growth factors may represent an adaptation to the oligotrophic waters found in the deep ocean.

The strains had varied responses to antibiotics (Table 6.1), ranging from strain Te-1-1 which was susceptible to all antibiotics tested, to strain Te-2-1, which was resistant to penicillin G, streptomycin, tetracycline, ampicillin and nalidixic acid.

All strains were strong reducers of both tellurite and selenite, resulting in the intracellular deposition of elemental Te and Se, respectively. The ability of each strain to resist and reduce metalloid oxides has been described (Rathgeber et al., 2002), and has not previously been reported for other *Pseudoalteromonas* species. Tellurite resistance tests for several closely related species in this study, showed high levels of resistance and

reduction (> 2000 μg per ml K_2TeO_3) for *P. distincta*, *P. undina*, *P. elyakovii*, *P. agarivorans*, and *P. espejiana* grown on Difco Marine Agar plates. *P. paragorgicola* was found to resist and reduce 100 μg per ml K_2TeO_3, and *P. atlantica* was able to resist 10 μg per ml K_2TeO_3 although reduction of the metalloid was not observed on any of the media tested.

6.4.3. DNA composition and phylogenetic analysis.

Sequence analysis of 16S rDNA was performed on three strains Te-1-1, Te-2-2^T and Se-1-2-red^T. The three isolates share high 16S rDNA similarities, ranging from 99.7 to 99.9%. Strains Te-1-1 and Se-1-2-red^T formed a cluster, along with strain Se-1-2-or (Rathgeber et al., 2002) (not evaluated in this study), within the γ-3 subclass of the *Proteobacteria*, with their closest known relative being *P. agarivorans*, which shares 99.5 to 99.8% 16S rDNA sequence similarity. Strain Te-2-2^T branched separately from the other three strains and was most closely related to *P. paragorgicola*, with 99.8% 16S rDNA similarity. A dendrogram presenting the phylogenetic relationship of isolates compared to other members of the genus *Pseudoalteromonas* is given in Fig. 6.2.

Comparison of the almost complete 16S rDNA sequences of Te-2-2^T and Se-1-2-red^T with the corresponding sequences of the phylogenetically closest type strains of *Pseudoalteromonas* species revealed similarities as high as 99.8%. Recent studies have indicated that for *Pseudoalteromonas* type strains, separated by 16S rRNA gene similarity levels as high as 99.9%, the corresponding DNA-DNA similarity values were clearly lower than 70%, which is the recommended threshold value for species delineation (Rathgeber et al., 2002). It thus appears that clear interstrain differences at the

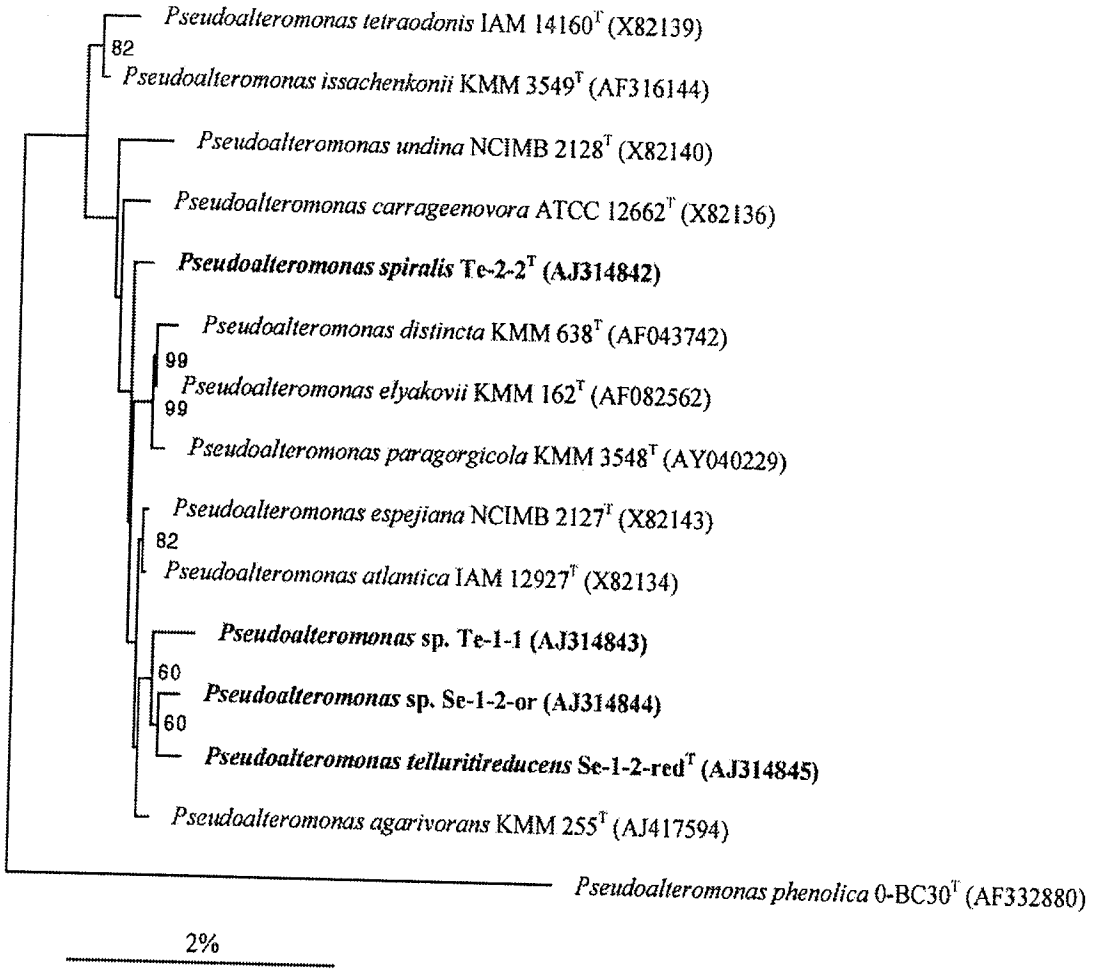

Fig. 6.2. Neighbour-joining dendrogram of 16S rDNA relatedness showing the position of *Pseudoalteromonas telluritireducens* strains Se-1-2-red^T and Te-1-1 and *P. spiralis* strain Te-2-2^T among other members of the genus *Pseudoalteromonas*. The scale bar represents 2 nucleotide substitutions per 100 sequence positions.

morphological and physiological level are more indicative for species differentiation than 16S rRNA sequence similarities. Riboprint analyses, using the enzyme EcoRI separates strain Te-2-2^T (DSM 16099^T) from *P. paragorgicola* DSM 14403^T (Fig. 6.3) and strain Se-1-2-red^T (DSM 16098^T) from *P. agarivorans, P. distincta, P. elyakovii, P. carrageenovora* and *P. espejiana*, with which this strain shares high 16S rRNA similarities. This restriction enzyme is not suitable to separate strain DSM 16099^T from the type strains of *P. undina* and *P. issachenkonii*. Good separation, however, was achieved with the enzyme *Pvu*II (Fig. 6.3).

The G+C contents determined for Te-1-1, Te-2-2^T and Se-1-2-red^T were 41.8, 39.6 and 39.9 mol%, respectively. These values are similar to those of other *Pseudoalteromonas* representatives which typically range from 37 to 50 mol% G+C (Gauthier et al., 1995).

Because of the high 16S rDNA sequence similarity of these isolates as well as the high similarity between all known members of the genus *Pseudoalteromonas* (greater than 98.5% identity) their taxonomic status is difficult to evaluate solely on this basis. However physiological differences (Table 6.2) such as the presence of a strong catalase activity, the inability to hydrolyze gelatin, and the broad temperature and pH ranges over which growth occurs, as well as habitat of isolation and differences in riboprint patterns justify the recognition of our rod shaped isolates (Te-1-1 and Se-1-2-red^T) as a new species, *Pseudoalteromonas telluritireducens*.

The interesting spiral shaped morphology of the second group of isolates (Te-1-2, Te-2-1, Te-2-2^T) along with the characteristics mentioned above, clearly allows for the creation of a new species *Pseudoalteromonas spiralis*.

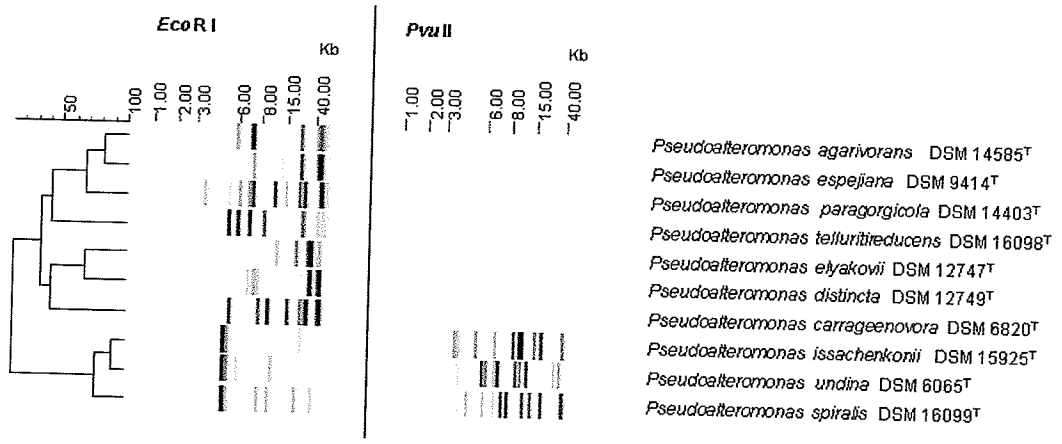

Fig. 6.3. Diversity of normalized ribotype patterns found in selected type strains of *Pseudoalteromonas*, related to strains Se-1-2-red^T (DSM 16098^T) and Te-2-2^T (DSM 16099^T). Cluster analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA) method based on the Pearson correlation coefficient, using an optimization coefficient of 1.24 (BioNumerics, Kortrijk, Belgium).

Table 6.2. Phenotypic characteristics distinguishing strains Te-2-2^T and Se-1-2-red^T from the most closely related species and from the type species *Pseudoalteromonas haloplanktis*.

Characteristic	Strain/Species					
	Te-2-2^T	Se-1-2-red^T	*P. carrageenovora**	*P. paragorgicola*†	*P. agarivorans*‡	*P. haloplanktis**
Cell shape	Vibrioid to spiral	Rod	Rod	Rod	Rod	Rod
G + C content (mol%)	39.6	39.9	39.5	41.1	42.2 – 43.8	41.8 – 44.4
Growth at °C						
4	+	+	-	+	-	-
37	+	+	-	-	ND	-
Growth in NaCl (%) 10.0	+	+	-	-	-	-
Utilization of						
Acetate	+	-	+	ND	-	+
Pyruvate	-	+	+	ND	ND	+
Fructose	-	+	+	ND	-	V
Glucose	+	+	+	ND	W	+
Citrate	+	+	+	-	-	+
Hydrolysis of						
Starch	-	+	-	+	+	V
Gelatin	-	+	+	+	+	+
Agar	-	+	-	-	+	-

+, substrate is utilized, substrate is hydrolyzed; -, substrate is not utilized, substrate is not hydrolyzed; V, varies between strains; ND, data not available. Data from *, (Mikhailov et al., 2001); †, (Ivanova et al., 2002); ‡, (Romanenko et al., 2003).

6.5. Description of *Pseudoalteromonas telluritireducens* sp. nov.

P. telluritireducens (tel.lu.ri'ti.re.du'cens. N. L. n. *telluris(-itis)* tellurite, L. part. adj. *reducens* reducing, N. L. part. adj. *telluritireducens* tellurite reducing, referring to the ability first discovered in this species of *Pseudoalteromonas*).

Gram negative, rod shaped bacterium (0.7 to 0.9 × 1.4 to 1.9 μm), motile by unknown means. Requires NaCl for growth, and can grow at concentrations of NaCl up to 10%, optimum growth occurs between 1.5 and 8.0% NaCl. Grows over a broad range of pH: 5.0 – 5.5 to 10.0 – 11.0, growing optimally between pH 5.5 and 9.5. Tolerates a wide range of temperatures, growth occurs as low as 4°C and as high as 45°C, although optimum growth occurs between 28 – 35°C. Catalase and oxidase positive. Hydrolyzes Tween 60 and starch, gelatin and agar. Obligately aerobic chemoorganotroph. Does not reduce nitrate. Reduces high levels of TeO_3^{2-} to Te(0) and SeO_3^{2-} to Se(0) aerobically resulting in black accumulations of Te or dark orange to red accumulations of Se, respectively. Glucose, fructose, glutamate, pyruvate, butyrate and citrate are used as the sole source of carbon and energy; may also utilize acetate, malate, succinate, lactate and ethanol, dependant on strain. Does not utilize formate or methanol. Does not require addition of organic growth factors. Antibiotic susceptibility is variable.

The G+C content of strain Se-1-2-red^T is 39.9 mol%.

The habitat is marine, deep ocean, in the vicinity of deep ocean hydrothermal vents.

The type strain is Se-1-2-red^T (DSMZ=16098^T = VKM B-2382^T). Other strains are: Te-1-1.

6.6. Description of *Pseudoalteromonas spiralis* sp. nov.

P. spiralis (spi.ra'lis. L. adj. *spiralis* spiral).

Gram negative, vibrioid to spiral shaped bacterium (0.6 to 0.7 × 3.2 to 5.3 μm), motile by means of a sheathed flagellum. Requires NaCl for growth, and can grow up to concentrations of 10% NaCl, optimum growth occurs between 0.5 and 10% NaCl. Grows over broad range of pH and temperatures, from pH 5.5 to 10.0 – 11.0 and from 4°C to 37 – 45°C, respectively, with optimal growth between pH 6.0 and 8.0 and at temperatures between 28 and 35°C. Catalase and oxidase positive. Hydrolyzes Tween 60, does not hydrolyze gelatin or agar, starch hydrolysis is variable. Obligately aerobic chemoorganotroph. May ferment fructose under microaerophillic conditions, does not reduce nitrate. Reduces high levels of TeO_3^{2-} to Te(0) and SeO_3^{2-} to Se(0) aerobically resulting in black accumulations of Te or dark orange to red accumulations of Se, respectively. All strains utilize acetate, glutamate and glucose as sole carbon and energy source. Pyruvate, citrate, malate, succinate, lactate and fructose may also be used. Butyrate, formate, ethanol and methanol are not utilized. Does not require organic growth factors, antibiotic susceptibility is variable.

The G + C content of the DNA is 39.6 mol%.

The habitat is marine, deep ocean, in the vicinity of deep ocean hydrothermal vents.

The type strain is Te-2-2^T (DSMZ=16099^T = VKM B-2383^T). Other strains are Te-1-2, Te-2-1.

6.7. Acknowledgements.

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Chapter 7.

Vertical distribution and characterization of aerobic phototrophic bacteria at the Juan de Fuca Ridge in the Pacific Ocean.

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Applied and Environmental Microbiology

(Submitted)

The material presented in Chapter 7 is derived primarily from experiments completed by Christopher Rathgeber. Coauthors contributing to this paper were Micheal T. Lince and Robert Blankenship, who performed the phylogenetic analysis; Jean Alric, who calculated the kinetics of cytochrome oxidation, fitted the redox titrations to Nernst curves and assisted with flash induced absorbance spectra; Elaine Humphrey, who prepared thin sections and assisted with electron microscopy; André Verméglio, who advised me on the biophysics experiments; F. Gerald Plumley, who determined the carotenoid compositions; Andrew S. Lang and Cindy L. Van Dover, who assisted with the collection of samples; J. Thomas Beatty who helped with cruise preparations and Vladimir Yurkov, who guided the project.

7.1. Abstract.

The vertical distribution of culturable anoxygenic phototrophic bacteria was investigated at five sites at or near the Juan de Fuca Ridge in the Pacific Ocean. Twelve similar strains of obligately aerobic phototrophic bacteria were isolated in pure culture, from depths ranging from 500 to 2379 m below the surface. These strains appear morphologically, physiologically, biochemically and phylogenetically similar to *Citromicrobium bathyomarinum* strain JF-1, a bacterium previously isolated from hydrothermal vent plume waters. Only one aerobic phototrophic strain, C23, was isolated from surface waters. This strain is morphologically and physiologically distinct from the strains isolated at deeper sampling locations, and phylogenetic analysis indicates that it is most closely related to the genus *Erythrobacter*. Phototrophs were cultivated from three water casts taken above vents but not from two casts taken away from active vent sites. No culturable anaerobic anoxygenic phototrophs were detected. The photosynthetic apparatus was investigated in strain JF-1 and contains light-harvesting I and reaction center complexes, which are functional under aerobic conditions.

7.2. Introduction.

Until the 1980s, anoxygenic bacterial phototrophy was thought to be confined to illuminated zones of anaerobic environments such as anoxic lakes, and sulfide springs, as production of the bacterial type photosynthetic apparatus is generally inhibited under aerobic conditions (Pfennig, 1978a). This belief led to a general assumption that anoxygenic phototrophy was unimportant in aerobic marine environments. However, the discovery of obligately aerobic BChl *a* producing bacteria in 1979 (Shiba et al., 1979;

Shiba and Simidu, 1982), indicated that anoxygenic phototrophy may be possible in a variety of aerobic environments. In recent years BChl-containing aerobes have indeed been isolated from a number of unusual environments and characterized, including marine and freshwater habitats, acid mine drainage sites, saline and soda lakes, and urban soils (Yurkov and Csotonyi, 2003; Rathgeber et al., 2004). Obligately aerobic BChl *a* producing bacteria, commonly known as APB (Beatty, 2002; Rathgeber et al., 2004) are a group of aerobic chemoorganoheterotrophs, possessing an anoxygenic photosynthetic apparatus, which functions only in the presence of oxygen (Rathgeber et al., 2004; Yurkov and Beatty, 1998a). Photosynthesis is estimated to satisfy up to 20% of cellular energy requirements under illuminated aerobic conditions (Yurkov and Van Gemerden, 1993b; Kolber et al., 2001). If the expression of the photosynthetic apparatus increased in response to nutrient deprivation, it would allow the organism to generate a portion of its energy via facultative photoheterotrophy, so as to better compete with other heterotrophs when nutrients become limited.

Although anoxygenic photosynthesis has historically been considered to be unimportant in marine environments, much recent research has focused on the presence of APB in the ocean. Biophysical results based on a fluorescent transient at 880 nm ascribed to bacterial anoxygenic light driven electron transport indicate that BChl *a*-containing bacteria may contribute up to 2-5% of the total photosynthetic electron transport fluxes in the upper ocean (Kolber et al., 2000), and suggest that APB account for up to 11% of the total marine heterotrophic population in the oligotrophic upper ocean (Kolber et al., 2001). Reports on culturability of *Erythrobacter*-like (Koblizek et al., 2003) and *Roseobacter*-like bacteria (Oz et al., 2005) from a variety of marine surface

waters including the Pacific, Atlantic and Indian Oceans, and the Mediterranean Sea support these data. However direct measurements of BChl by HPLC (Goericke, 2002) and anoxygenic phototroph abundances calculated by IREM and quantitative PCR (Schwalbach and Fuhrman, 2005) indicate that APB account for much less of the total heterotrophic population in marine systems. These reports have focused primarily on anoxygenic phototrophy in the upper ocean. Vertical measurements of BChl *a* by HPLC and direct microscopic counts of BChl containing cells by IREM indicate that APB numbers decrease rapidly below about 75 m depth in the oligotrophic Pacific Ocean (Kolber et al., 2001).

Perhaps the most unusual report was of the BChl-containing strain JF-1, later named *Citromicrobium bathyomarinum*, isolated from hydrothermal vent plume waters of the Juan de Fuca Ridge, in the Pacific Ocean (Yurkov and Beatty, 1998b;Yurkov et al., 1999). It has been suggested that near infrared blackbody radiation emitted at deep ocean vents may provide sufficient energy to power phototrophic metabolism (Nisbet et al., 1995, Van Dover et al., 1996), and indeed the culture of an obligately anaerobic phototroph from effluent plume waters at the East Pacific Rise has recently been reported (Beatty et al., 2005). The publication of *C. bathyomarinum*, JF-1 not only stimulated active research on APB in open ocean surface waters (discussed above), but also raised a number of questions from the scientific community. Two major questions that needed answers were: 1. Was JF-1 endemic to black smoker plumes or distributed throughout the water column? 2. Was JF-1 able to use infrared light emitted by black smokers for photosynthesis?

This paper reports on the cultivability of APB from five sites at or near the Juan de Fuca Ridge in the Pacific Ocean. We show that pure cultures of strains phylogenetically related and phenotypically similar to *C. bathyomarinum* are readily obtainable from depths of 500 m and below, and that they possess a functional photosynthetic apparatus consisting of a RC and a single LH I complex.

7.3. Materials and methods.

7.3.1. Collection of samples.

Water samples were collected over a ten day period in the vicinity of the Juan de Fuca Ridge in the Pacific Ocean in July, 2000. Samples were taken using 20 l Niskin bottles mounted on board a CTD rosette sampler, from depths of 0, 500, 1000, 1500, 2000 m and from 10 m above the sea floor at five different sites. Coordinate locations of the five sampling sites are given in Table 7.1. Fifty ml samples were removed from the Niskin bottles after cleaning the nozzle with 95% ethanol and flushing approximately 1 l of sample water through ethanol cleaned plastic tubing. Samples were immediately used to inoculate growth media.

7.3.2. Isolation.

Serial dilutions were performed using a medium for purple non-sulfur bacteria modified from Pfennig (1978b) (PNS) containing in g l^{-1}: KH_2PO_4, 0.3; NH_4Cl, 0.3; KCl, 0.3; $CaCl_2$, 0.05; NaCl, 20.0; Na acetate, 1.0; malic acid, 0.3; yeast extract, 0.2;

Table 7.1. CTD cast sampling locations.

Sampling location	Lat.	Long.	Depth (m)	Description
1	47°57.0N	129°05.8W	2164	Above Hulk*, North Main Endeavour
2	47°57.0N	129°05.8W	2176	Above Hulk , North Main Endeavour
3	47°57.0N	129°08.5W	2375	Offset from Hulk
4	48°27.6N	128°42.8W	2379	Above Middle Valley
5	48°34.3N	129°58.4W	>2500	Open ocean

*, geological formation Hulk described in Delaney et al., 1992.

supplemented with 2 ml of a trace element solution (Drews, 1983) and 2 ml of a vitamin solution (Yurkova et al., 2002), autoclaved at pH 5.9 and adjusted to pH 7.8 – 8.0 by addition of 0.5 N NaOH. Diluted samples were plated onto nutrient rich RO NaCl plates (Yurkov et al., 1999) for growth of copiotrophs and a nutrient poor modified CHU 10 medium (Gerloff et al., 1950) for growth of oligotrophs, containing in g l^{-1}: $Ca(NO_3)_2$, 0.232; KH_2PO_4, 0.01; $MgSO_4$, 0.025; Na_2CO_3, 0.02; $NaSiO_3$, 0.044; $FeCl_3$, 0.0025, supplemented with 2 ml of the above trace element and vitamin solutions, autoclaved, and adjusted to pH 8.0 after sterilization. RO NaCl plates were incubated in the dark at room temperature for 20 days prior to enumeration. CHU 10 plates were incubated at room temperature in front of a window (daylight) for 20 days. After incubation plates were enumerated, and representative colonies were transferred into liquid RO NaCl media, and streaked repeatedly on RO NaCl plates until pure cultures were achieved.

Purified strains that formed pigmented colonies were grown at 30 °C on agar plates overnight in the dark, and cells were resuspended in 150 µl 10 mM Tris-HCl, pH 7.8 and mixed with 450 µl of a 30% BSA solution (ICN Biomedicals) to reduce light scattering. Absorbance spectra were recorded between 350-1100 nm (Hitachi U-2010 spectrophotometer). The presence of a characteristic LH absorbance peak at approximately 870 nm was used to infer the presence of BChl *a*.

Agar deeps were prepared using three media types. PNS (given above) supplemented with 5 ml l^{-1} each of filter sterilized 0.3 mM cysteine and methionine solutions. A purple sulfur bacteria medium (PS) containing in g l^{-1}: KH_2PO_4, 0.3; NH_4Cl, 0.3; $CaCl_2$, 0.05; NaCl, 20.0 and 2 ml each of the above trace element and vitamin solutions, adjusted to pH 5.9 before autoclaving. After sterilization the following were

added in ml l^{-1}: 0.3 mM cysteine, 5; 0.3 mM methionine, 5; 10% $NaHCO_3$, 20; 10% Na_2S, 3.5; and the pH was adjusted to 7.8-7.9. A pyruvate mineral salts (PMS) medium containing in g l^{-1}: EDTA, 0.1; $MgSO_4$, 0.2; $CaCl_2$, 0.075; NH_4Cl, 1.0; K_2HPO_4, 0.9; KH_2PO_4, 0.6; Na pyruvate, 2.2; NaCl, 20.0; yeast extract, 0.1; with 2 ml each of the above trace element and vitamin solutions, autoclaved at pH 6.8. Following sterilization, 5 ml l^{-1} each of 0.3 mM cysteine and 0.3 mM methionine were added and the pH was adjusted to 7.5-7.8. The media were mixed 2:1 with molten 2% agar containing 20.0 g l^{-1} NaCl, to achieve a final concentration of 0.67% agar. Deeps were allowed to cool to about 45°C before inoculation with 1 ml of diluted samples, and then closed with a rubber stopper to exclude oxygen. After solidification, agar deeps were stored on ice until return to the lab (5-15 days) where they were incubated at 30°C in an illuminated incubator (light intensity of ~ 30 $\mu E\ m^{-2}\ s^{-1}$).

7.3.3. Physiology and biochemistry.

Physiological and biochemical tests were performed as previously described (Yurkov and Van Gemerden, 1993a; Yurkov et al., 1994b). Photoheterotrophic growth under anaerobic conditions was tested in screw cap tubes containing RO NaCl, PNS or PS media (Rathgeber et al., 2005).

Pigments were extracted in acetone:methanol (7:2) and total carotenoid content was determined using an Agilent Technologies HPLC Model 1100 equipped with a Microsorb-MV (Rainin Instruments) C18 column (4.6 × 250 mm). The initial mobile phase was 75% acetone for 3 min and increased to 100% acetone over 15 min. Carotenoids were detected with the UV-VIS detector set to scan from 350-600 nm.

7.3.4. Microscopy.

Morphology and cytology were examined in log-phase cultures grown in liquid RO NaCl medium under dark aerobic conditions at 30°C, by phase contrast (Zeiss Axioskop 2) and electron microscopy (Hitachi H7600 TEM or Zeiss EM 10C TEM). Negative stains were performed by treating cells with 1.0% aqueous uranyl acetate. For thin sections, the bacteria were embedded in Epon after fixation with 2.5% glutaraldehyde and 1.0% osmium tetroxide (Kellenberger et al., 1958).

7.3.5. Phylogenetic analysis.

Extraction of genomic DNA, PCR amplification of 16S rRNA gene segments and sequence analyses were performed as previously reported (Rainey et al., 1996). Sequences were aligned using a sequence alignment editor (Bioedit). Phylogenetic trees were constructed using the algorithms contained in TREECON for Windows, and bootstrap values were determined using the TREECON package.

7.3.6. Photosynthetic apparatus.

The methods of membrane and LH-RC complex isolation by sucrose density gradient centrifugation were previously described (Yurkov et al., 1993; Yurkov et al., 1994a). Cells were grown in a minimal glucose medium containing in g l^{-1}: $MgSO_4$, 0.5; KH_2PO_4, 0.3; NH_4Cl, 0.3; KCl, 0.3; $CaCl_2$, 0.05; NaCl, 20.0; glucose, 1.0; yeast extract, 0.05, adjusted to pH 5.5 and incubated at 28°C with shaking in the dark. The photosynthetic electron transport study and titrations of the RC primary electron donor

and primary electron acceptor were performed as reported by Yurkov et al. (Yurkov et al., 1998a; b)

7.4. Results and discussion.

7.4.1. Isolation.

To answer the question about endemism of *C. bathyomarinum*, water samples were collected in the vicinity of the Juan de Fuca Ridge from different depths including the surface (see section 7.3). One hundred sixty-three strains of yellow, bright yellow, citron-yellow, peach, pink and red pigmented, aerobic heterotrophic bacteria (representative of all pigmented morphotypes present on RO NaCl and CHU 10 plates) were isolated in pure culture and screened for the presence of BChl. Of these 163 strains, 13 strains similar in color, bright-yellow to citron-yellow, were found to produce BChl *a*. Table 7.2 describes sampling location and depth for each strain.

Interestingly, BChl *a*-containing strains were found throughout the water column at sites directly above deep ocean hydrothermal vent fields, although no such strains were found at sites offset from the vents (Table 7.1 and 7.2). Isolates of APB were obtained from all depths tested, and although only one strain originated from surface waters, it should be noted that total heterotroph numbers were far higher (5-10X) in samples taken from the surface than from deep waters. This may have directly influenced our ability to culture APB using these methods. Heterotrophs could out-compete APB on the relatively rich medium and thus overcrowd agar plates, preventing development of slower growing APB colonies. Additionally, production of antagonistic compounds by several

Table 7.2. Isolates of aerobic BChl *a* containing bacteria.

Strain	Depth (m)	Site[1]	Medium	Dilution[2]
C6	1500	1	CHU 10	10^{-1}
C7	1500	1	CHU 10	10^{-1}
C46	1500	1	CHU 10	10^{-1}
C8	2164	1	CHU 10	10^{-1}
N25	2164	1	RO	10^{-1}
C12	500	2	CHU 10	10^{-1}
C14	500	2	CHU 10	10^{-1}
N34	500	2	RO	10^{-1}
N78	1000	2	RO	10^{-1}
C23[3]	Surface	4	CHU 10	10^{-1}
C26	1500	4	CHU 10	10^{-2}
N56	1500	4	RO	10^{-1}
N48	2379	4	RO	10^{-1}

[1], See Table 7.1 for site location. [2], Dilution at which strain was isolated. [3], All strains form citron-yellow colonies, except C23, which is bright-yellow.

marine bacteria including some *Pseudoalteromonas* species has been reported (Holmstrom and Kjelleberg, 1999). Presumably colonies that produce antimicrobial agents, grown on an agar surface, would inhibit the growth of other bacterial species, including APB.

Anaerobic agar deeps were incubated for 30 days at 30 °C under continuous illumination, resulting in the growth of non-pigmented colonies. No pigmented phototrophic colonies were detected in the three media tested, which however does not definitively confirm their absence in samples, as the majority of species in any given environment are frequently considered to be uncultivable. Beja et al. (2002) reported the discovery of photosynthesis gene clusters most closely related to those found in α, β and γ-*Proteobacteria* through culture-independent studies of DNA sequences recovered from Monterey Bay and from the central North Pacific Ocean. At the present time all but one known phototroph, *Roseateles depolymerans* (Suyama et al., 1999), belonging to the β-*Proteobacteria* (Rathgeber et al., 2004), and all known phototrophs of the γ-*Proteobacteria* display a typical anaerobic anoxygenic photosynthetic metabolism. Therefore the discovery of photosynthesis genes closely related to those found in β- and γ-*Proteobacteria* suggest that anaerobic phototrophs are indeed present in ocean surface waters.

7.4.2. Phenotypic and physiological properties.

To recognize the taxonomical relationship of new strains to *C. bathyomarinum*, a set of taxonomical identification tests had to be performed and thirteen aerobic yellow BChl *a* producing strains were isolated in pure culture and subjected to further

characterization. All had similar absorbance characteristics with an *in vivo* BChl peak at 865-867 nm indicative of a photosynthetic LH I complex, and a peak at 801-802 nm indicative of a RC. The spectral properties of these strains correlate well with that of *C. bathyomarinum* JF-1 which also has a relatively small amount of BChl giving rise to peaks at 867 and 800 nm (Yurkov et al., 1999). The magnitude of the absorbance peaks attributed to BChl in new isolates is extremely small in comparison to that typically seen for APB (Fig. 7.1). Comparative absorbance spectra of strain C8 and *Erythrobacter litoralis*, strain T4, show that deep ocean BChl containing organisms produce far less photosynthetic units than the most commonly cultured APB (Fig. 7.1).

Absorbance spectra also show peaks attributed to the presence of carotenoids at 434, 457-458 and 487 nm in all strains (Fig. 7.1) again in accordance with those of *C. bathyomarinum* (Yurkov et al., 1999). Analysis by HPLC revealed the major carotenoid present in 12 isolated strains and JF-1 to be erythroxanthin sulphate. Interestingly strain C23, the only isolate from surface waters, possessed caloxanthin sulphate as the major carotenoid, even though the spectral characteristics are identical to that of the deep ocean strains.

Four of the strains (C6, C8, C23, C46) as well as *C. bathyomarinum*, JF-1 were tested for their ability to utilize organic substrates, hydrolyze certain macromolecules and for their tolerance to high levels of NaCl (Table 7.3). All strains were tolerant to a wide range of salinity with robust growth occurring from 0 to 8% NaCl. Reduced growth occurred up to 14% NaCl for C23, and up to 16% for the other strains. The new isolates utilized a somewhat greater variety of organic substrates than JF-1 (Yurkov et al., 1999). Strains C6, C8, C23 and C46 grew heterotrophically on acetate, puruvate, glutamate,

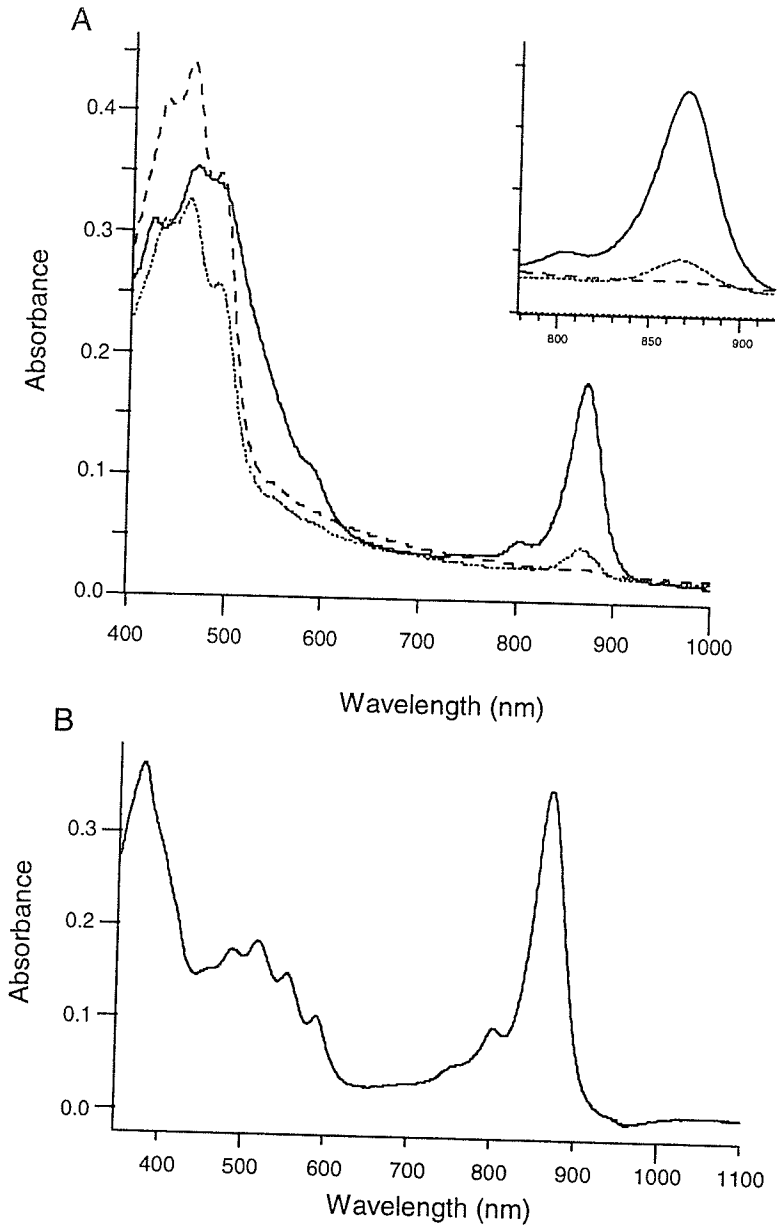

Fig. 7.1. Absorption spectra of (A) strain JF-1 grown in rich organic medium (dashed line) and in minimal glucose medium at pH 5.5 (dotted line) show a peak at about 865 nm indicating the presence of BChl incorporated into a light-harvesting I complex. The spectrum of *E. litoralis* strain T4 (solid line) is given for comparison. Inset shows the 800-900 nm region in greater detail. (B) Partially purified light-harvesting-reaction center complexes of strain C8 (see text).

Table 7.3. Comparative characteristics of deep ocean aerobic phototrophs.

Test	Strain				
	JF-1	C6	C8	C23	C46
Growth at NaCl %					
0	++	++	++	++	++
2	++	++	++	++	++
4	++	++	++	++	++
6	++	++	++	++	++
8	++	++	++	++	++
10	+	++	+	+	+
12	+	+	+	+	+
14	+	+	+	W	+
16	W	W	W	-	W
20	-	-	-	-	-
Organic sources					
Acetate	W	+	+	+	+
Pyruvate	-	+	+	+	+
Glutamate	+	+	+	+	+
Butyrate	+	++	++	++	++
Malate	-	-	-	-	-
Lactate	-	-	-	-	-
Citrate	-	-	-	-	-
Succinate	-	-	-	+	-
Formate	-	-	-	-	-
Glucose	W	++	++	++	++
Fructose	-	-	-	-	-
Ethanol	-	+	+	+	+
Methanol	-	-	-	-	-
Yeast extract	+	++	++	++	++
Hydrolysis of					
Gelatin	+	+	++	+	+
Starch	-	-	W	-	W
Tween 60	+	+	++	W	+
Major carotenoid pigment	Erythroxanthin sulfate	Erythroxanthin sulfate	Erythroxanthin sulfate	Caloxanthin sulfate	Erythroxanthin sulfate

+, positive; -, negative; W, weak positive.

butyrate, glucose and ethanol as sole source of carbon. Additionally C23 is capable of growth on succinate. All strains hydrolyze gelatin and Tween 60, as does JF-1, additionally C23 and C46 exhibit weak hydrolysis of starch.

None of the strains grew under anaerobic conditions in the presence or absence of light. BChl *a* was produced only under dark aerobic conditions as revealed by characteristic absorbance in the 800 and 870 nm regions, which is typical for APB (Yurkov and Beatty, 1998a).

Morphology and cytology were examined in three selected strains (C6, C8 and C46). Cells of each strain were similar, consisting of short (0.6 × 1.6 μm) to long (0.6 × 2.4 μm) Gram-negative, motile rods, which occurred singly or in rosette-like formations (Fig. 7.2a), and often formed Y-shaped cells (Fig. 7.2b). The cytoplasmic membrane was visible, but no ICM of the type usually implicated to hold the photosynthetic apparatus in typical anaerobic anoxygenic phototrophs were detected. The absence of obvious ICM is characteristic of APB and it is suggested that the poorly developed photosynthetic apparatus is located directly in the cytoplasmic membrane (Yurkov and Beatty, 1998a). No inclusions or storage materials were observed. The mode of cell division varies as budding (Fig. 7.2c), binary and trinary fission (Fig. 7.2b) were detected. As in JF-1, negative stains show the production of as yet unknown cell connective materials (Fig. 7.2d), and reveal the presence of one or more polar to sub-polar flagella.

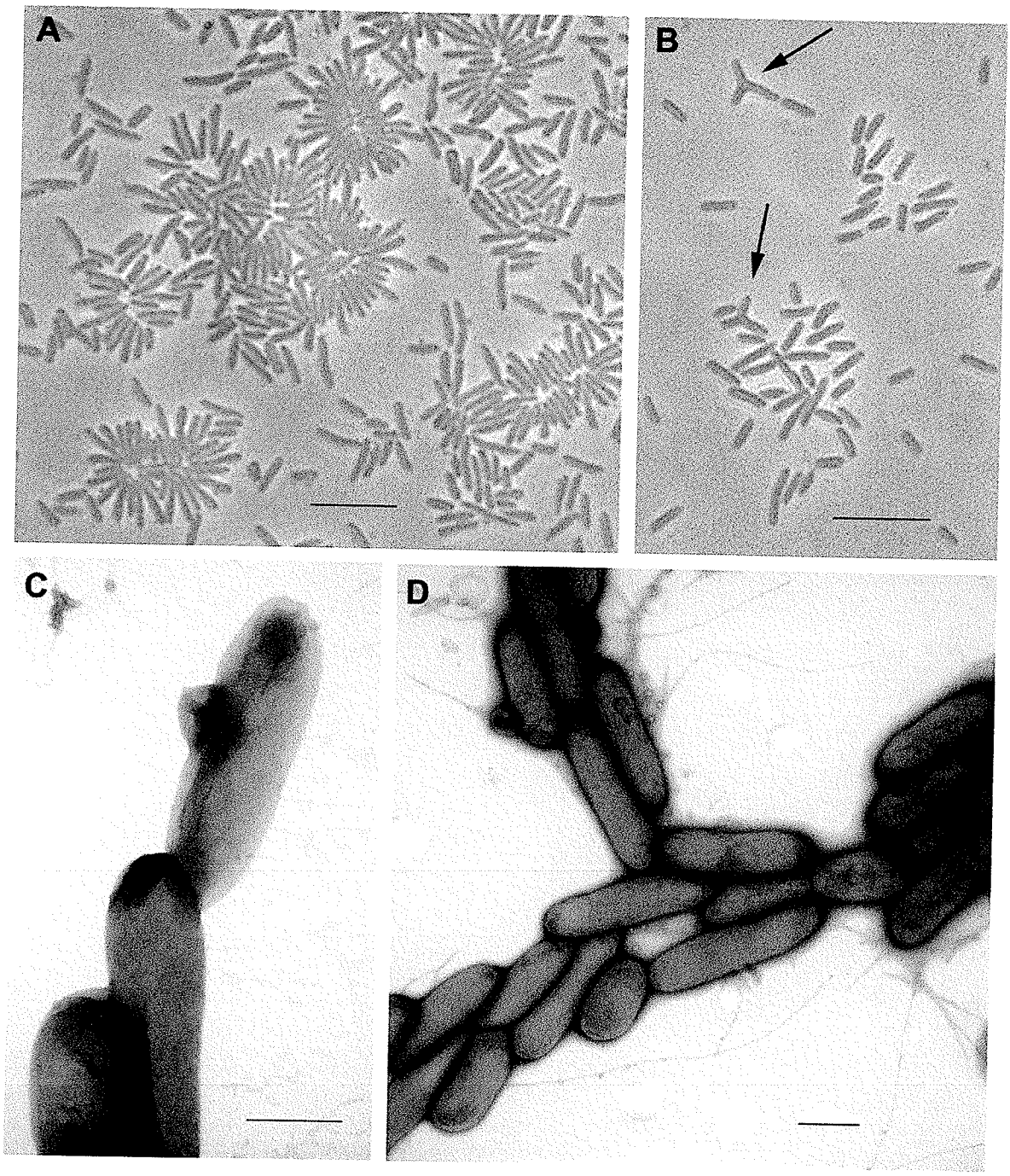

Fig. 7.2. Phase contrast microscopy of strain C8 shows (A) the formation of interesting rosette-like conglomerates of cells, and (B) pleomorphism, characteristic of the deep ocean *C. bathyomarinum*, including the production of "Y-cells" (indicated by arrows). Electron microscopic negative stains of (C) strain C6 show replication by budding, and (D) connective materials produced by strain C46. Bars: (A, B) 5 μm, (C, D) 500 nm.

7.4.3. Phylogenetic analysis.

To determine phylogenetic relationships and to confirm a genospecies identity with *C. bathyomarinum*, genomic DNA was extracted from eight BChl *a* containing strains. Analysis of almost complete (1391 nucleotides) 16S rRNA genes shows that seven deep ocean strains (C6, C8, C14, C46, N25, N48, N78) are closely related to *C. bathyomarinum*, JF-1 (99.7-99.8% sequence similarity). They form a distinct grouping with strain JF-1 supported by 100% bootstrap confidence, which confirms their identity as members of this species (Fig. 7.3). Strain C23, the lone phototrophic isolate from surface waters is more distantly related to C. *bathyomarinum*, falling within the *Erythrobacter-Porphyrobacter-Erythromicrobium* cluster of the class *Alphaproteobacteria*, related most closely to *E. litoralis* (96.9%). This suggests that C23 is a new species within the genus *Erythrobacter*.

7.4.4. Photosynthetic apparatus.

As it is stated in the introduction, the capability of *C. bathyomarinum* to use infrared light emitted by black smokers and its photosynthetic competence in general were at the center of polemic discussions. To solve this puzzle, we selected the type strain of the species, JF-1, and the newly isolated C8 for study of the photosynthetic apparatus.

Because production of BChl in JF-1 was increased when cells were grown in a minimal glucose medium at pH 5.5 (Fig. 7.1a), these conditions were used for all further experiments. The increased production of BChl observed in minimal media agrees well with the idea that APB use photosynthesis as an auxiliary energy source under nutrient-

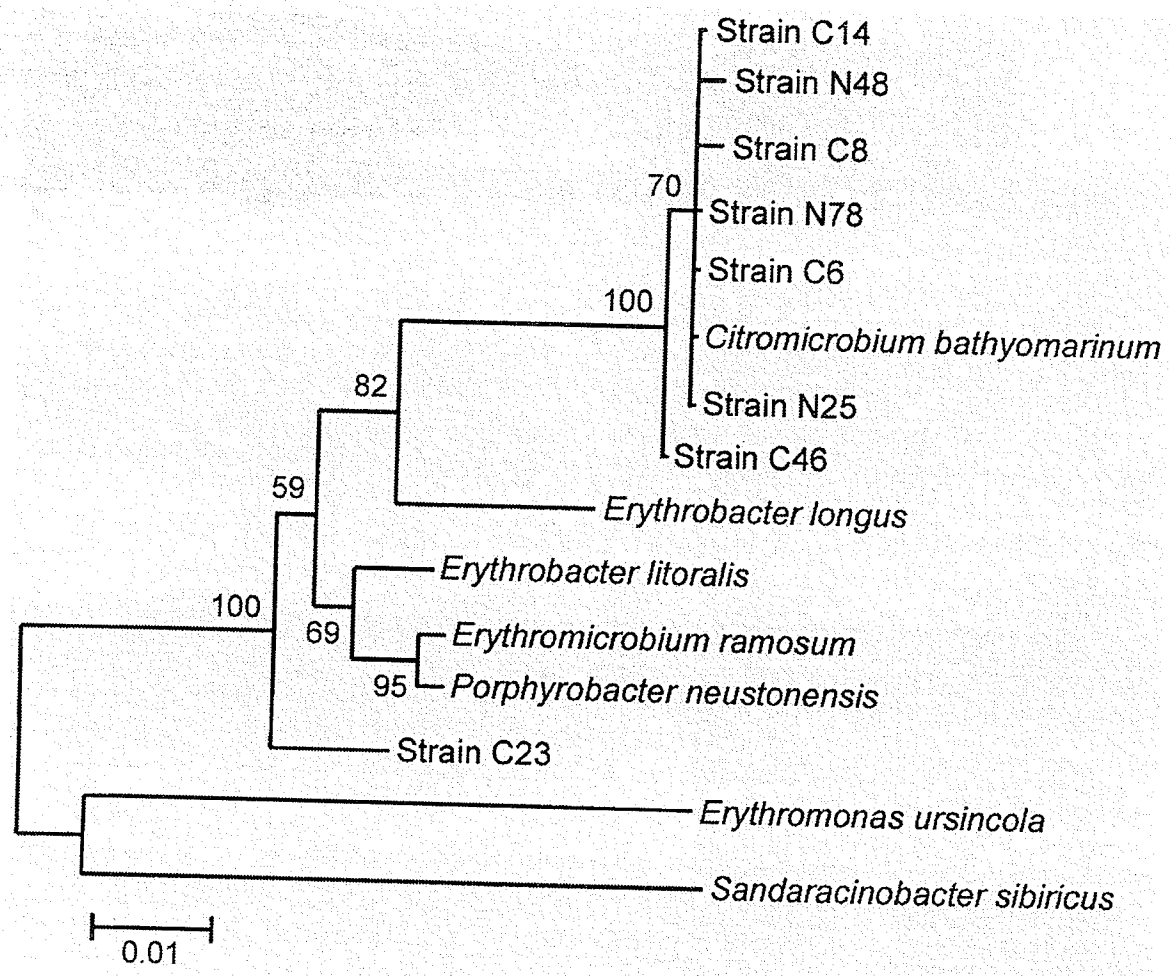

Fig. 7.3. Unrooted phylogenetic tree showing the position of eight isolated strains among the most closely related aerobic phototrophic species, within the α-4 subclass of the *Proteobacteria*. Bootstrap values, based on 500 resamplings, are indicated. Bar, 1 substitution per 100 sequence positions.

deprived (oligotrophic) conditions, allowing the organism to satisfy a portion of its energetic requirements using light energy and thus allowing it to better compete with non-phototrophic heterotrophs.

To confirm the organization of the photosynthetic apparatus in *C. bathyomarinum*, photosynthetic units from strains JF-1 and C8 were isolated after disruption of cells using a French press, treatment of photosynthetic membranes (PSM) with detergent and subsequent sucrose density gradient centrifugation. Fractions containing the LH-RC complex were further purified by anion exchange chromatography. Absorbance spectra of purified fractions confirmed the presence of a LH I complex with a major absorbance peak at 867 nm and a photosynthetic RC peak at 801 nm (Fig. 7.1b). No evidence of a LH II complex was found.

To gauge the ability of *C. bathyomarinum* to carry out photosynthetic electron transport and thus produce cellular energy from light, we examined photoinduced electron transfer in JF-1 whole cells, photosynthetic membrane fragments and purified LH-RC complexes. Although photochemistry was clearly observed in both whole cells and membrane fragments, the signal strength was weak. This is likely due to the great amounts of yellow carotenoids, absorbing robustly in the blue light region, and the relatively low quantity of photosynthetic units produced by JF-1. Light induced absorbance changes recorded in whole cells of JF-1, taken under aerobic conditions (Fig. 7.4) showed absorbance troughs at both 555 nm and 605 nm, 50 μs after an excitation flash, indicating a fast electron transfer between the RC and a cyt (Yurkov et al., 1998b). This trough at 555nm shifted to 552 nm by 7 ms after excitation. Absorbance spectra measured in membrane fractions and purified LH-RC complexes have the same trough

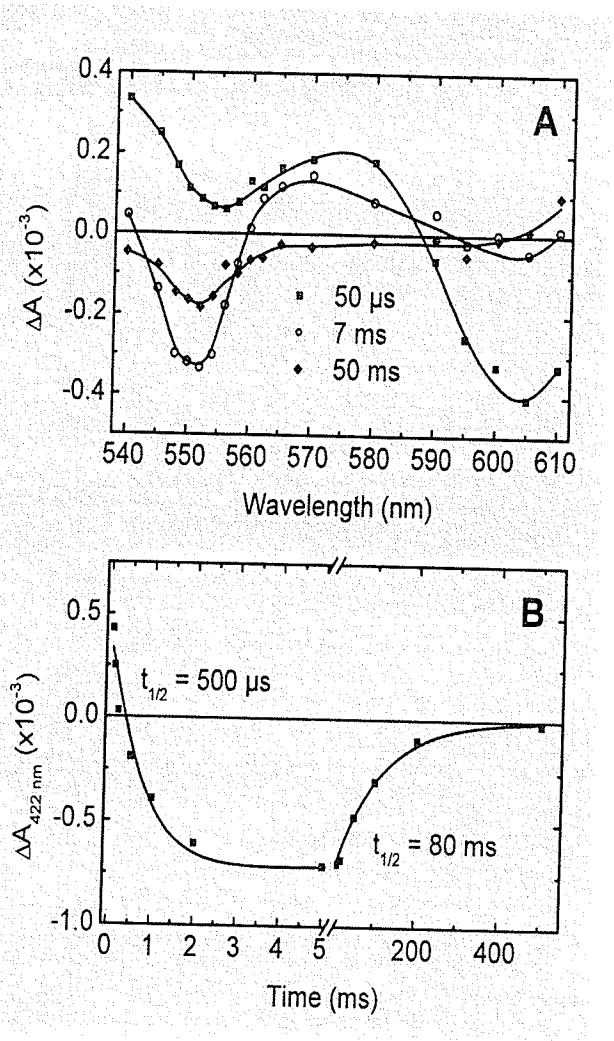

Fig. 7.4. (A) Flash-induced absorbance spectra of JF-1 whole cells measured under aerobic conditions. The trough observed at 555 nm, 50 µs after excitation, is attributed to photooxidation of a RC-bound cyt. The shift to 552 nm observed at 7 ms is attributed to the oxidation of a soluble cyt, indicating light-induced cyclic electron transfer. ΔA, change in absorbance. (B) A kinetic of electron transfer demonstrates re-reduction of the primary electron donor and subsequent re-reduction of the bound cyt. $\Delta A_{422\ nm}$, change in absorbance measured at 422 nm.

at 555 nm after 50 μs, although the shift to 552 nm is absent at 7 ms, presumably due to the absence of a soluble cyt *c* (Yurkov et al., 1998b). The absorbance changes observed in whole cells are attributed to the reduction of the RC-bound cyt. This RC-bound cyt displays unusual activity concerning the re-reduction of the RC primary e^- donor, presenting a half-time of about 500 μs (Fig. 7.4), which is very slow for a re-reduction by a RC-bound tetraheme cyt, which usually occurs within a few μs (Shopes et al., 1987). Once oxidized, the rate of re-reduction for the soluble cyt (presumably by the cyt bc_1 complex) displays a halftime of about 80 ms, indicating that under aerobic conditions JF-1 is indeed capable of photosynthetic cyclic e^- transfer. The rate of this cyclic e^- transfer is slow but is comparable to the rate of cyclic e^- transfer found in some anoxygenic phototrophs (Shopes et al., 1987). Under anaerobic conditions the case is different. Absorbance changes in the 552-555 nm region were not observed after an excitation flash, meaning that the e^- carriers did not undergo redox changes and photosynthetic e^- transfer did not occur. From this set of experiments we can conclude that *C. bathyomarinum* can be photosynthetically active if infrared light is available.

To understand why photosynthetic e^- transfer occurs only under aerobic conditions the redox potentials of the RC primary e^- donor (P/P^+) and primary e^- acceptor (Q_A/Q_A^-) were titrated in membrane fractions (Fig. 7.5). The midpoint redox potential of the Q_A/Q_A^- was found to be + 80 mV versus a standard hydrogen electrode and the midpoint potential of the P/P^+ was around + 470 mV. Previously it was demonstrated that the photosynthetic e^- transfer system of other APB such as *Roseobacter denitrificans*, *E. litoralis*, *Erythromicrobium ursincola*, *Sandaracinobacter sibiricus* and

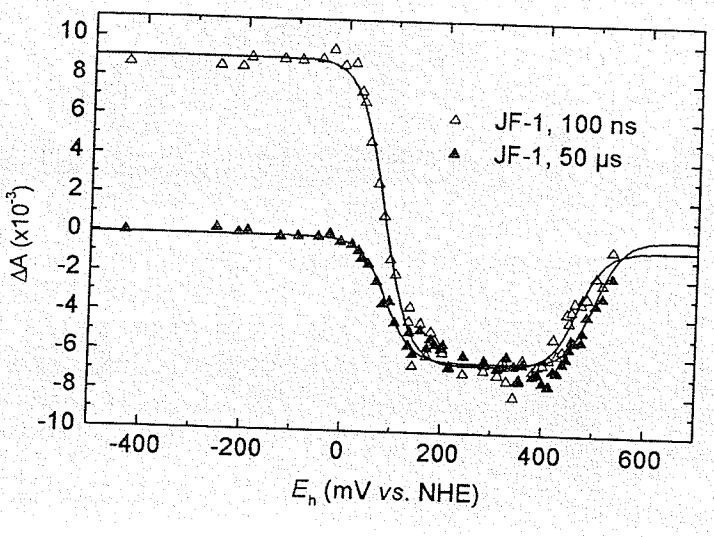

Fig. 7.5. Redox titration of the reaction center primary electron donor (P/P^+) and primary electron acceptor (Q_A/Q_A^-) performed on membrane fraction of strain JF-1. ΔA, light induced absorbance changes were measured at 605 nm. NHE, standard hydrogen electrode.

Roseococcus thiosulfatophilus is inoperative in anaerobic cells, presumably due to the high midpoint potential of Q_A (Yurkov and Beatty, 1998a). This rather high midpoint potential of Q_A explains why there is no charge separation under anaerobic conditions, and thus, may be one of the reasons why *C. bathyomarinum*, as well as other APB, is incapable of anaerobic photosynthetic growth.

In summary, we show that strains of *C. bathyomarinum* are readily cultured from deep ocean waters above the Juan de Fuca Ridge in the Pacific Ocean. The inability to successfully culture APB from deep ocean waters at sites offset from hydrothermal vents remains a mystery. Possibly nutrients ejected in vent effluents rise through the water column, thus supporting a different microflora in those particular locations. The presence in deep ocean waters and absence of *Citromicrobium*-like APB in surface waters despite the detection of *Erythrobacter*-like strains suggest that the habitat of *C. bathyomarinum* is indeed endemic to the lightless deep ocean. However some factors, including competition by other heterotrophs and overcrowding of nutrient rich agar plates by the abundance of microbial cells in surface waters, might account for our inability to isolate *C. bathyomarinum* from those samples.

The only APB that we isolated from surface waters, strain C23, shares several physiological properties with *C. bathyomarinum*, however it has a different pigment composition and is phylogenetically distinct, appearing more closely related to *Erythrobacter*, a genus also isolated from marine surface environments. The low levels of BChl produced by C23 (as well as all strains of *C. bathyomarinum)*, and the effects of culture conditions on BChl production, suggest that identification of APB from the

Pacific Ocean should include a thorough analysis of pigments lest strains producing low or variable amounts of BChl be overlooked.

7.5. Aknowledgements.

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Chapter 8.

Description of the photosynthetic apparatus, and photoinduced electron transfer in the aerobic phototrophic bacteria *Roseicyclus mahoneyensis* and *Porphyrobacter meromictius*.

Christopher Rathgeber, Jean Alric, André Verméglio and Vladimir Yurkov

(To be submitted)

The material presented in Chapter 8 is derived primarily from experiments completed by Christopher Rathgeber. Coauthors contributing to this paper were Jean Alric, who calculated the kinetics of cytochrome oxidation, fitted the redox titrations to Nernst curves and assisted with flash induced absorbance spectra; André Verméglio, who advised me on the biophysics experiments, and assisted with fluorescence measurements and Vladimir Yurkov, who guided the project.

8.1. Abstract.

Photosynthetic electron transfer has been examined in whole cells, isolated membranes and in partially purified reaction centers of *Roseicyclus mahoneyensis*, strain ML6^T and *Porphyrobacter meromictius*, strain ML31, two new species of obligate aerobic anoxygenic phototrophic bacteria. Photochemical activity in strain ML31 was observed aerobically, but the photosynthetic apparatus was not functional under anaerobic conditions. In strain ML6^T low levels of photochemistry were measured anaerobically, possibly due to partial reduction of the primary electron acceptor (Q_A) prior to light excitation, however, electron transfer occurred optimally under low oxygen conditions. Photoinduced electron transfer involves a soluble cytochrome *c* in both strains, and an additional reaction center bound cytochrome *c* in ML6^T. The redox properties of the primary electron donor (P) and Q_A of ML31 are similar to those previously determined for other aerobic phototrophs, with midpoint redox potentials of +463 and -25 mV, respectively, and are higher than found in anaerobic phototrophs. Strain ML6^T showed a very narrow range of ambient redox potentials appropriate for photosynthesis, with midpoint redox potentials of +415 mV for P and +94 mV for Q_A. Cytoplasm soluble and photosynthetic complex bound cytochromes were characterized in terms of apparent molecular mass. Fluorescence excitation spectra revealed that abundant carotenoids not intimately associated with the reaction center are not involved in photosynthetic energy conservation.

8.2. Introduction.

Obligate aerobic anoxygenic phototrophs synthesize BChl *a* incorporated into photosynthetic RC and LH complexes, however, unlike those found in typical anoxygenic phototrophic bacteria, these pigment protein complexes are incapable of light induced e^- transfer under anaerobic conditions (Yurkov and Beatty, 1998). In earlier studies, it was found that e^- transfer in APB could be distinguished into two separate types based on the mode of the photooxidized primary e^- donor (P^+) re-reduction. The first one involved a cyt *c* intimately bound to the RC, allowing for very fast re-reduction of the P^+. Such e^- transfer has been observed in *Erythromonas ursincola*, *Sandaracinobacter sibiricus*, *Roseococcus thiosulfatophilus* and *Roseobacter denitrificans* (Garcia, et al., 1994; Yurkov et al., 1998b; Yurkov and Beatty, 1998). In the second type, found in all tested species of *Erythromicrobium* and in *Erythrobacter longus*, no RC-bound cyt was present and P^+ was re-reduced through transfer of an e^- directly from a soluble cyt *c* (Yurkov et al., 1998b). There was also no RC-bound cyt in *Erythrobacter litoralis*, and oxidation of the soluble cyt *c* was relatively slow, further differentiating its photochemistry from other APB (Yurkov et al., 1998b). In each of these cases the path of e^- transfer closely resembles those discovered in anaerobic phototrophic bacteria (Yurkov and Beatty, 1998), however the photosynthetic e^- transport chain in APB was catalytically active only under relatively oxidized conditions (Yurkov and Beatty, 1998; Rathgeber et al., 2004). A number of speculations have been proposed to explain this difference. 1) The relatively high redox potential of the Q_A observed in APB (*E. litoralis*, *E. ursincola*, *S. sibiricus*, *R. thiosulfatophilus* and *R. denitrificans*) (Yurkov et al., 1998a; Rathgeber et al., 2004), results in fully reduced Q_A under anaerobic

conditions, leaving it incapable of accepting an e^- and participating in e^- transport (Rathgeber et al., 2004). 2) The redox state of cyt in the e^- transport chain may be responsible for the lack of photochemistry anaerobically (Garcia et al., 1994). Under reduced conditions the low potential hemes of the *R. denitrificans* RC-bound tetraheme cyt were capable of re-reducing P^+, but the hemes may not be re-reduced by the soluble cyt *c* (Schwarze et al., 2000; Rathgeber et al., 2004). Absence of e^- transfer from the soluble cyt to the low potential hemes of the RC-bound cyt results in an incomplete e^- transfer chain. However, this speculation could not be applied to all APB and immediately raised the question: What about species that do not produce a tightly bound tetraheme cyt in the RC? Does the redox potential of a soluble cyt restrict e^- transfer anaerobically? Or is there an alternative low potential soluble cyt that is capable of re-reducing P^+, but cannot itself be re-reduced by the cyt bc_1 complex? Therefore the major scientific question in regards to the name-sake feature of APB (the inability to grow anaerobically by photosynthesis) remains without a confirmed answer.

Currently, anoxygenic phototrophic bacteria fall into two groups: conventional anoxygenic phototrophs that grow robustly under illuminated anaerobic conditions, and APB that photosynthesize only in the presence of O_2. The evolution of the photosynthetic apparatus, from anaerobic to aerobically active complex, remains unexplained. It has been suggested that APB may have evolved to fill a specific niche (Beatty, 2002), or that lateral transfer of photosynthesis genes may have occurred, giving rise to APB by "accident". The heterogeneity of the photosynthetic apparatus is not yet fully understood, and it is still not known if there exists an intermediate evolutionary stage between anaerobic phototrophs and APB, an organism capable of photosynthesis independent of

the presence or absence of O_2. Further study of photosynthesis in new and diverse species is essential to solve these dilemmas.

Roseicyclus mahoneyensis, ML6^T and *Porphyrobacter meromictius*, ML31 are interesting examples of APB recently discovered in the meromictic Mahoney Lake in British Columbia, Canada (Yurkova et al., 2002). *R. mahoneyensis*, phylogenetically related to the α-3-*Proteobacteria*, is unusual in its tendency to form almost cyclical-shaped cells, and to produce a rare type of LH II, previously known only in the aerobic *Roseobacter* and *Rubrimonas* (Rathgeber et al., 2005) and the anaerobic purple non-sulfur bacterium *Rhodopseudomonas palustris* (Hartigan et al., 2002). The monomodal LH II (LL B800) of *R. palustris* is controlled by a bacteriophytochrome and produced only under low light conditions (Evans et al., 2005), however, this has not been shown for *R. mahoneyensis*, in which BChl *a* production is strongly inhibited by light (Rathgeber et al., 2005). *P. meromictius* is a halotolerant prosthecate bacterium, branching within the α-4-*Proteobacteria*, which produces a relatively large quantity of BChl as compared to other APB (Yurkova et al., 2002; Rathgeber et al. 2006). In the current study we examine the organization and function of the photosynthetic apparatus of these species to better understand the aspects of photosynthesis in APB.

8.3. Materials and methods.

8.3.1. Growth conditions.

Batch cultures of *R. mahoneyensis* were grown at 30°C in a dark shaking incubator (170 rpm) in medium N1 (Yurkova et al., 2002). *P. meromictius* was grown under the same conditions using a modification of medium N1 containing in $g \cdot l^{-1}$: $MgSO_4$, 2.0; KH_2PO_4, 0.3; NH_4Cl, 0.3; KCl, 0.3; $CaCl_2$, 0.05; $NaSO_4$, 50.0; Na-acetate, 1.0; adjusted to pH 7.8 – 8.0 and supplemented with 2 ml each of vitamin and trace element solutions (Yurkova et al., 2002).

8.3.2. Isolation of soluble cyt, membranes and pigment protein complexes.

Cells were harvested at the end of the exponential growth phase, washed with 10 mM Tris-HCl buffer, pH 7.8 and concentrated by centrifugation. To 100 ml of a thick cell suspension were added: 1 ml of 10% EDTA in Tris-HCl, pH 7.8; 1 ml of 1 mM PMSF in 95% ethanol; 0.05 g of DNAase (Fisher Scientific). Cells were then disrupted by three passes through a French Press at 20 K PSI. Unbroken cells and debris were removed by centrifugation at 12 K × g for 20 min. The supernatant was centrifuged again at 150 K × g for 12 h (strain ML31) or 15 h (strain $ML6^T$) to separate soluble from membrane fractions. The supernatant was reserved and soluble cyt were collected by passing the soluble fraction through a 100 kDa YM ultrafiltration membrane under a stream of nitrogen.

Cell membranes were homogenized in 10 mM Tris-HCl, pH 7.8 and purified by sucrose density gradient centrifugation (0.9 M – 1.8 M sucrose in 10 mM Tris- HCl, pH

7.8) at 38 K rpm for 15-16 h. Individual bands were removed from centrifuge tubes using Pasteur pipettes and absorbance spectra were measured to identify PSM (presence of BChl peaks at 800 and 870 nm), which were collected, centrifuged at 150 K × g to sediment and re-suspended in fresh buffer.

Pigment protein complexes were isolated from PSM of ML31 by treatment with 0.6% LDAO at room temperature for 20 min in the dark followed by sucrose density gradient centrifugation (0.2 – 0.9 M sucrose in 10 mM Tris-HCl, pH 7.8 containing 0.05% LDAO) at 38 K rpm for 15-16 h. Colored bands were removed and absorbance spectra measured to identify fractions containing LH-RC complexes. Sucrose and LDAO were removed by three 8 h dialyses in Tris-HCl, pH 7.8 and concentrated by packing dialysis tubing in polyethylene glycol-6000 for 2 h. The LH-RC complexes of ML31 were relatively unstable when purified and partially degraded into RC and free BChl after dialysis (see section 8.4). These RC were further purified by anion exchange chromatography on a DEAE cellulose column equilibrated with Tris-HCl, pH 7.8 (0.05% LDAO added). Fractions containing RC were collected and dialyzed to wash and concentrate, as above.

Membranes of strain $ML6^T$ were treated with 8% Triton X-100 at room temperature for 30 min to release LH-RC complexes followed by sucrose density gradient centrifugation (0.3 – 1.0 M sucrose in 10 mM Tris-HCl, pH 7.8; 0.05% Triton X-100) and processed as described for ML31.

8.3.3. Analytical methods.

The presence of cyt and their molecular weights were determined by SDS-PAGE on a 15% acrylamide/bis-acrylamide gel or on an 11-17% linear gradient acrylamide/bis-acrylamide gel, stained using 3,3',5,5'-tetramethylbenzidine (TMBZ) in the presence of hydrogen peroxide as described (Thomas et al., 1976).

8.3.4. Spectrophotometric procedures.

Absorbance spectra were recorded at room temperature using a Hitachi U-2010 spectrophotometer. Light induced absorbance spectra were measured in whole cells, PSM and pigment protein complexes using equipment similar to that described by Joliot et al. (1980), with excitation provided by xenon flash bulbs. Redox titrations of P/P^+ and Q_A/Q_A^- were performed using whole PSM suspended in 10 mM Tris-HCl, pH 7.8. Excitation was provided by xenon flashes as described (Yurkov et al., 1998a). Fluorescence excitation spectra were calculated by measuring fluorescence between 830 to 890 nm produced by excitation over a series of wavelengths from 350 to 650 nm.

8.4. Results and discussion.

8.4.1. Isolation of membranes and purification of pigment protein complexes.

Membranes were separated from cells of both strains, ML31 and $ML6^T$. PSM of ML31 appeared as a dark-red band migrating to approximately 1.7 M sucrose, whereas membranes of $ML6^T$ appeared dark-purple and migrated to about 0.5 M sucrose. Both strains produced only pigment protein complex containing membranes. Other

membranes, not containing BChl, were not detected. Considering the APB characteristic lack of an ICM system (Yurkov and Beatty, 1998; Rathgeber et al., 2005; Rathgeber et al., 2006), this confirms that the photosynthetic apparatus is indeed located directly in the cytoplasmic membrane. This differs from results found for *Erythromicrobium ramosum* and *R. thiosulfatophilus*, which produced two distinct membrane fractions (Yurkov et al., 1994). The LH-RC complexes were located mainly (*R. thiosulfatophilus*), or wholly (*E. ramosum)* in one fraction indicating a discontinuous organization of the membrane despite the lack of obvious ICM.

The LH-RC complexes of ML31 were separated by treatment of dense membrane suspensions with 0.6% LDAO at room temperature, resulting in liberated LH-RC that migrated to 0.7 M sucrose during density gradient centrifugation. The LH-RC fraction was collected and dialyzed to remove sucrose and detergent (Fig. 8.1a). After dialysis, most LH-RC complexes had degraded leaving RC intact and free BChl of the decomposed LH I. This is unusual as a second, stronger detergent treatment is typically required to release RC from the LH (Yurkov et al., 1998a), and indicates that the LH antennae of ML31 is very fragile, unstable in solution and may be less tightly associated with the RC than seen in other APB. The liberated and still intact RC component was further purified by anion exchange chromatography on a DEAE cellulose column equilibrated to pH 7.5. The RC collected after chromatography were stable in a 10 mM Tris-HCl solution at pH 7.8, containing 0.05% LDAO (to solubilize complexes) and could be stored at -20 °C (Fig. 8.1b).

Membranes of strain ML6^T proved extremely resilient to detergent, remaining intact after treatment with concentrations not higher than 0.6% LDAO. Treatment with

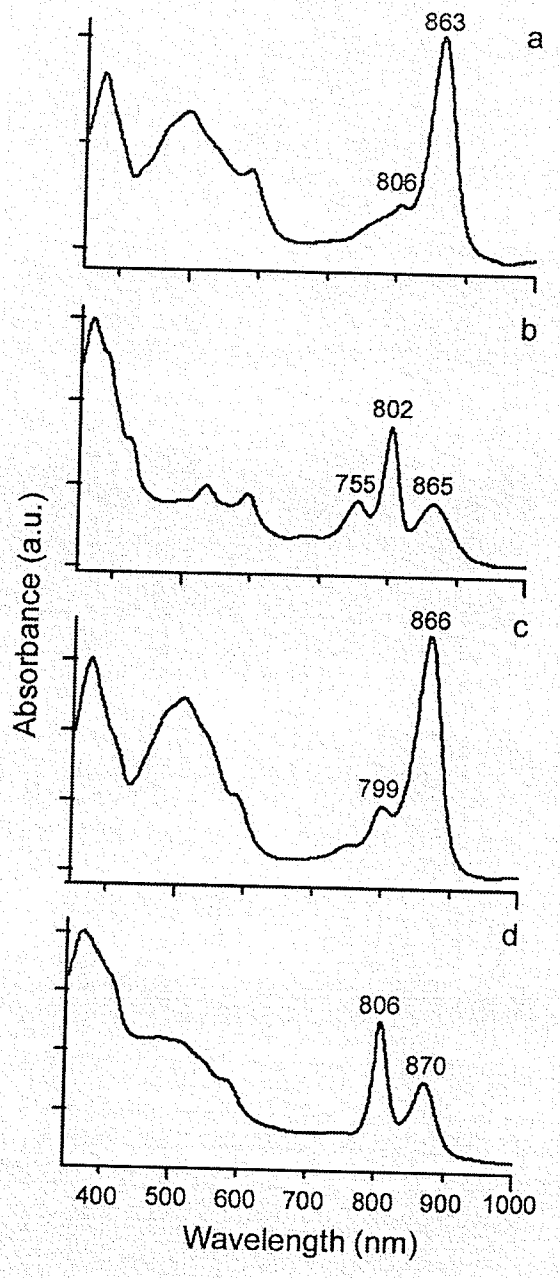

Fig. 8.1. Absorbance spectra of ML31 (a) partially purified reaction center-light harvesting I complexes, (b) reaction centers, and (c) $ML6^T$ reaction center-light harvesting I complexes, suspended in 10 mM Tris-HCl pH 7.8 containing 0.05% LDAO. (d) Intact cells of $ML6^T$ show light harvesting complex I (870 nm) and the monomodal light harvesting complex II (806 nm).

1% LDAO solubilized membranes but resulted in the destruction of pigment protein complexes, leaving only small amounts of intact RC-LH I contaminated with large amounts of free BChl. However, solubilization of membranes using 8% Triton X-100 incubated for 30 min at room temperature was sufficient to solubilize membranes while leaving RC-LH I complexes intact. The unusual monomodal LH II (805-806 nm) of $ML6^T$ (Fig. 8.1d) (Rathgeber et al., 2005) was very sensitive to both detergents tested, and degraded to BChl. Such instability of *Roseicyclus*' monomodal LH II was unexpected because spectrophotometrically similar monomodal LH II (805 nm) of *Roseobacter* resisted purification procedures (Shimada et al., 1985). The difference may arise from variations in amino acid sequence, alignment factors or from specific interacting configurations of the LH I components. The detergent resistant RC-LH I complexes were further purified through sucrose density gradient centrifugation (migrated to 0.3 M sucrose) followed by dialysis to remove sucrose and detergent. Final purification was achieved by DEAE cellulose column chromatography as described above, using 0.05% LDAO to maintain solubility of the proteins. The RC-LH I collected after chromatography remained stable in 10 mM Tris-HCl solution at pH 7.8 containing 0.05 % LDAO and could be stored at -20 °C (Fig. 8.1c).

8.4.2. Light induced e^- transfer.

Results in several laboratories have confirmed that the APB photosynthetic apparatus is active (Okamura et al., 1984; Garcia et al., 1994; Yurkov et al., 1998b) under oxidized conditions, and lack of photochemistry under reduced conditions is consistent with their inability to grow anaerobically by photosynthesis. Two major ideas have been

presented to explain such unusual behavior. 1) The redox potential of Q_A is relatively positive compared to typical anaerobic anoxygenic phototrophs, causing it to be reduced under anaerobic conditions. In this state the Q_A cannot accept e^- from P, preventing cyclic e^- transfer. This type of restriction has been observed in *R. denitrificans*, *E. litoralis*, *E. ramosum*, *E. ursincola*, *S. sibiricus* and *R. thisulfatophilus* (Yurkov and Beatty, 1998). 2) Low potential hemes of the bound cyt, in *R. denitrificans*, may reduce P^+ under reduced ambient conditions. However, the soluble cyt is unable to re-reduce the low potential hemes due to a higher redox potential, thus halting e^- transfer reactions (Schwarze et al., 2000). Nevertheless, it still remains unclear if such peculiarity is specific to the RC of *R. denitrificans*, because it cannot be applicable to all APB. If redox poise of the tetraheme cyt would be the only factor limiting anaerobic photosynthesis, then why is e^- transfer oxygen dependent in those species lacking a RC bound cyt?

We measured photo-induced absorbance difference spectra in whole cells, PSM and isolated pigment protein complexes of both strains to evaluate their potential for e^- transfer after excitation, and to determine the effect of oxygen on the ability to carry out photosynthesis. Under aerobic conditions the photooxidation of the P^+ in ML31 was observed 50 μs after an actinic flash confirmed by absorbance differences at 435 and 603 nm (Fig. 8.2a). In spectra measured 1 ms and 20 ms after excitation, these differences had been replaced by troughs at 422 and 552 nm, attributed to the oxidation of a *c* type cyt. The relatively slow re-reduction of the P^+ indicated that e^- were transferred directly from cyt *c* as has been demonstrated for APB of the genera *Erythromicrobium* and *Erythrobacter* (Yurkov et al., 1998b).

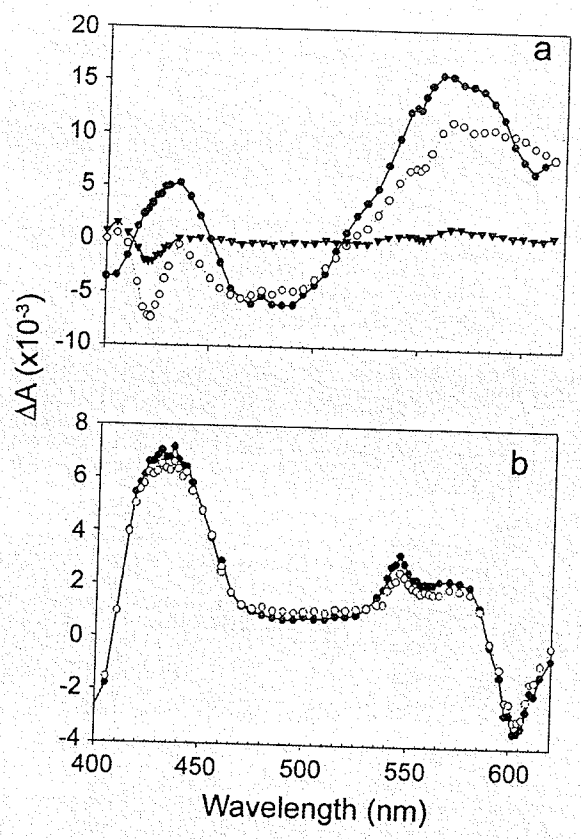

Fig. 8.2. Light induced absorbance difference spectra recorded in ML31 (a) whole cells under aerobic conditions taken at (●) 50 μs (○) 1 ms and (▼) 20 ms after excitation, and (b) membranes in the presence of 5 mM sodium ascorbate, (●) at 50 μs and (○) 500 ms after excitation.

To calculate the rate of e^- transfer, a kinetic of cyt oxidation was recorded, which showed the half time of e^- transfer between the soluble cyt and P^+ to be 170 μs and the half time of cyt re-reduction, 10 ms (Fig. 8.3a). The re-reduction rate was comparable to that reported in whole cells of the genus *Erythromicrobium* (Yurkov et al., 1998b) and *E. longus* (Okamura et al., 1984).

Light induced absorbance spectra of ML31 PSM under ambient (oxidized) conditions revealed a strong P^+ signal at 430 and 603 nm, 50 μs after excitation (Fig. 8.2b). This signal remained stable 500 ms after excitation, indicating absence of re-reduction and confirming that re-reduction of the P^+ should be mediated by a soluble cyt that had been washed out during membrane preparation and not by a tetrahemic RC bound cyt. When a similar experiment was performed under reduced conditions (addition of 5 mM sodium ascorbate), results were the same as seen under ambient conditions, supporting that the absence of P^+ re-reduction was not due to oxidation of the cyt before excitation. The absence of a RC bound cyt was further revealed in the absorbance spectra of membranes, and light induced difference spectra of isolated RC taken under both oxidized and reduced conditions. In absorbance spectra there was no change in characteristics due to the reduction of a cyt upon addition of either sodium ascorbate or dithionite. In light induced difference spectra a signal corresponding to oxidation of P^+ at 50 ms slowly faded over 200 ms, and no signal of cyt oxidation was detected (Fig. not shown).

According to light induced difference spectra, measured in whole cells of ML31 under anaerobic conditions, the P^+ remained capable of e^- transport functions, however the soluble cyt was incapable of re-reducing P^+. This is consistent with the strain's

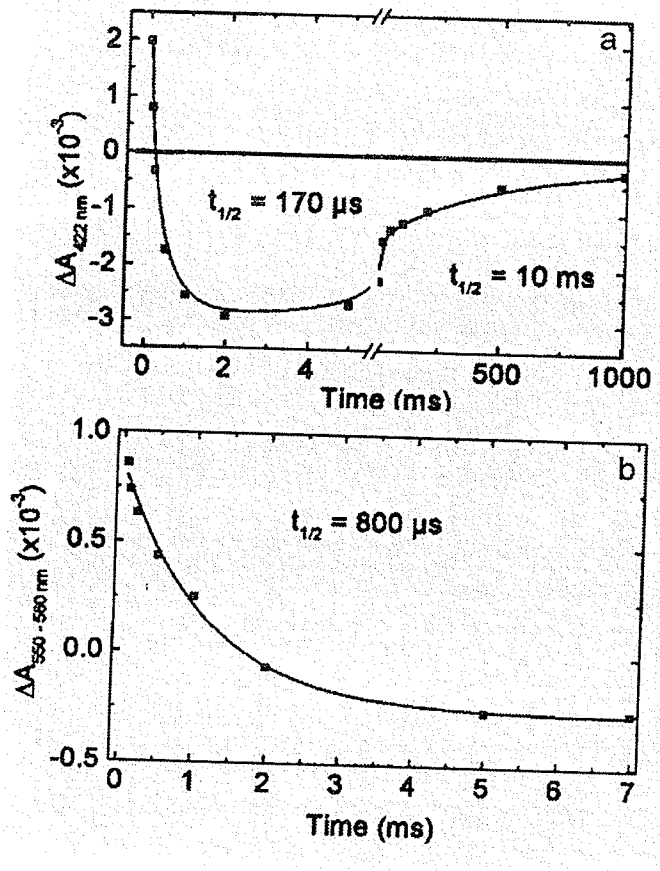

Fig. 8.3. Kinetic of soluble cyt oxidation in whole cells of (a) ML31, and (b) $ML6^T$ after excitation (time = 0), observed at 422 nm.

inability to photosynthesize under anaerobic conditions and is similar to behavior of other APB (Yurkov and Beatty, 1998; Rathgeber et al., 2004).

In whole cells of ML6^T, light induced difference spectra recorded under fully aerobic conditions revealed the presence of oxidized cyt (difference at 422 and 555 nm) superimposed on a signal due to an oxidized Q_A (603 nm) (Fig. 8.4a). Therefore the e^- transfer was inefficient, possibly caused by the partial oxidation of cyt before excitation. The inefficiency of e^- transfer resulted in a P^+ signal (603 nm) that remained stable 20 ms after excitation. When difference spectra were taken under anaerobic conditions, cells were capable of photosynthetic e^- transfer involving a very fast re-reduction of P^+, giving rise to a difference signal at 422 and 555 nm due to the oxidation of cyt 50 ms after excitation (Fig. 8.4b). The signal, however, was weak indicating that the Q_A may already be partially reduced because of the anaerobic conditions before the excitation flash.

To better understand the effect of oxygen on photosynthetic e^- transfer in ML6^T, we analyzed the degree of e^- transfer efficiency as a growing culture respired (and thus removed) available O_2. Flash induced absorbance changes were measured in intact cells within a closed cuvette. The active cells consumed O_2 from the medium resulting in its gradual decrease over time. When O_2 levels had been lowered below a certain threshold, the efficiency of photoinduced e^- transfer increased, reaching a maximal activity after 56 min of incubation. With longer incubation, and consistently lower O_2 concentrations, e^- transfer was gradually inhibited (Fig. 8.5). The pattern of changing efficiency of cyt photooxidation over the time of incubation was attributed to a narrow range of O_2 concentration that supported maximal photosynthetic e^- transfer in living cells, which presumably resulted in optimal redox poise of the e^- carriers. These data correlate with

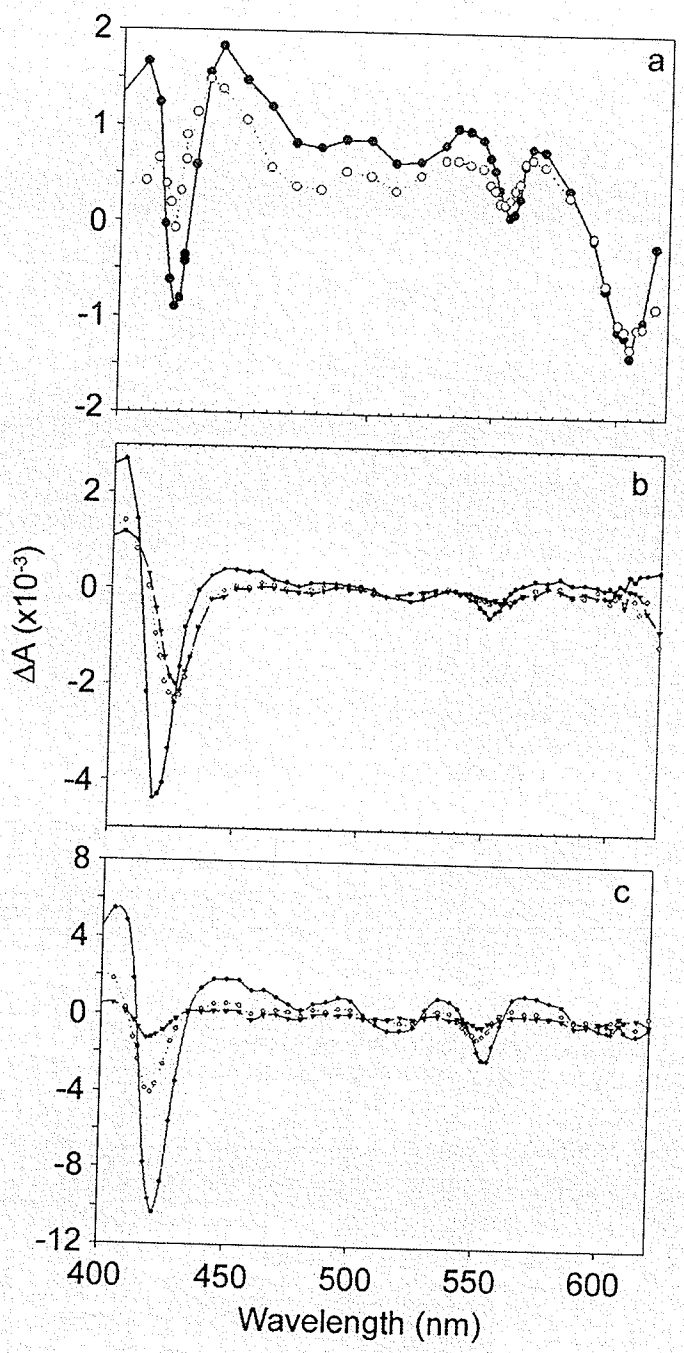

Fig. 8.4. Light induced absorbance difference spectra in ML6^T whole cells recorded under (a) aerobic, (b) anaerobic, and (c) partially aerobic conditions, (●) 50 μs, (○) 20 ms and (▼) 50 ms after excitation (b and c only).

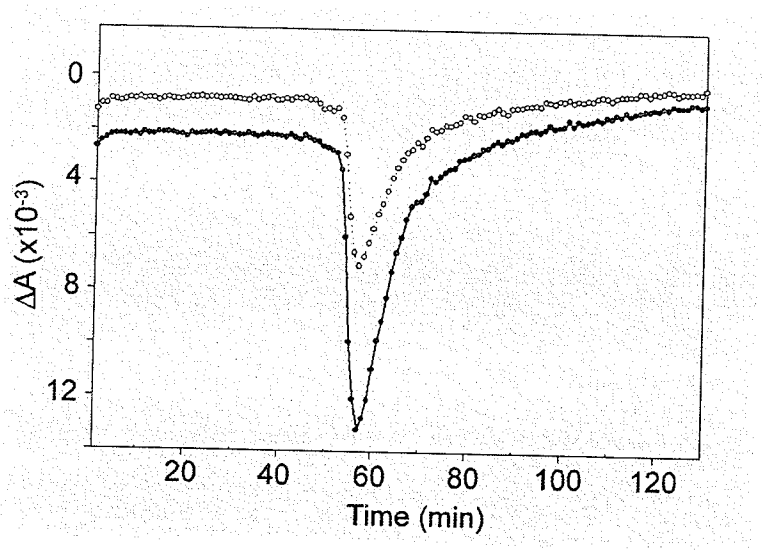

Fig. 8.5. Flash induced absorbance differences measured at 422 nm, recorded (●) 50 μs and (○) 20 ms after excitation every 1 min as cells respired oxygen from the cuvette.

the idea that the photosynthetic apparatus of $ML6^T$ is fully functional only under semi-aerobic conditions.

Flash induced experiments run on partially (instead of fully) aerobic cells yielded significantly different results. Partial aerobiosis was reached by waiting a short period of time for active cells to respire some of the O_2 to create microaerophillic conditions. A simulated partial aerobiosis could also be achieved by inhibiting respiration of the cells using a low concentration (2 mM) of KCN. In the partially aerobic state, light induced e^- transfer was maximal with clear differences due to cyt oxidation (422 and 555 nm) 50 μs after the flash (Fig. 8.4c). At this time point, P^+ signal was absent. The very fast rate of P^+ re-reduction, indicated the presence of a cyt bound tightly to the RC. At 20 ms after excitation, there was a shift in the signal from 555 to 552 nm, indicating e^- transfer from a soluble cyt. The signal then faded as the soluble cyt was re-reduced, presumably by the cyt bc_1 complex. When KCN was added, the rate of signal fading was significantly slower, as the bc_1 complex was likely no longer functional. The RC-bound cyt displayed unusual activity concerning the re-reduction of the P^+, with a half-time of about 800 μs (Fig. 8.3b), very slow for re-reduction by a RC-bound tetraheme cyt, which usually occurred within a few μs (Shopes et al., 1987).

The results were supported by measuring light induced difference spectra in $ML6^T$ membranes. Under ambient (oxidized) conditions e^- transfer reactions were absent. Cyt were oxidized before the flash resulting in a P^+ signal at 50 μs that stayed stable through 500 ms and no cyt oxidation peaks were noticed (Fig. 8.6a). Under reduced conditions (addition of 5 mM Na-ascorbate), e^- transfer reactions were partially functional, confirming the presence of a RC-bound cyt (troughs at 422 and 555 nm) (Fig.

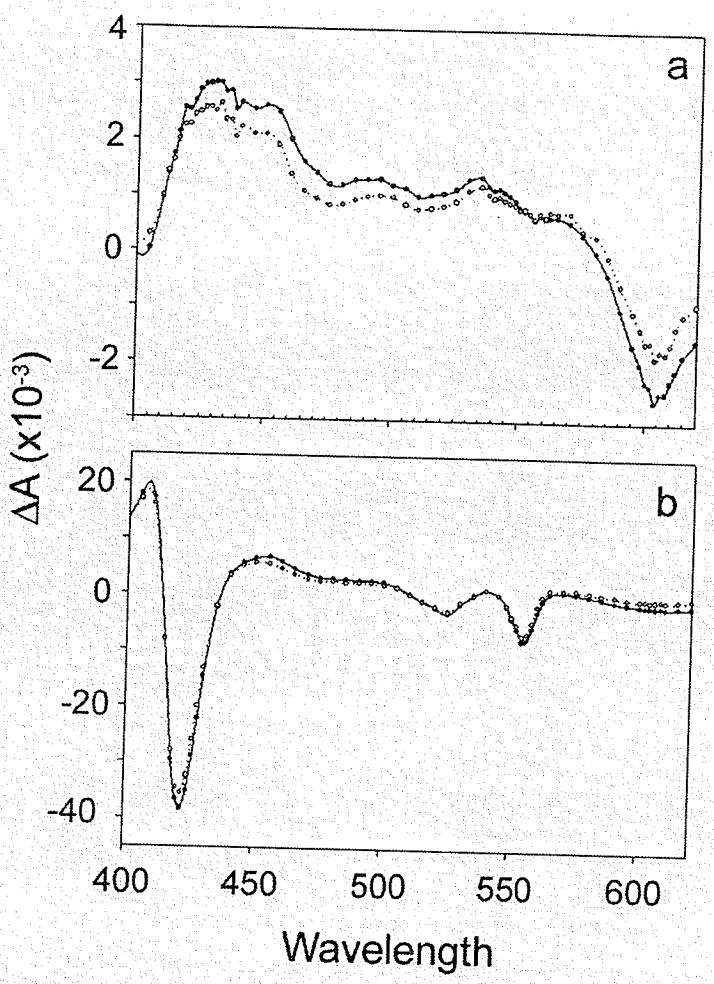

Fig. 8.6. Flash induced absorbance spectra of ML6^T membranes under (a) ambient conditions and (b) reduced conditions recorded (●) 50 μs and (○) 500 ms after excitation.

8.6b). Absence of a difference shift to 552 nm after 20 ms agreed with the shift seen in whole cells due to the RC-bound cyt re-reduction by a soluble cyt washed out in membrane preparations.

8.4.3. Redox potentials of the P^+ and Q_A.

To better understand the effect of oxygen on photoinduced e^- transfer, the redox potentials of the P^+ and Q_A were titrated in membranes. Flash induced spectral differences were recorded at 605 nm (to detect P^+) and measurements were taken 50 μs after excitation for ML31, and 100 ns and 50 μs for $ML6^T$, over a range of ambient redox potentials from about -300 to 490 mV versus a standard hydrogen electrode. ML31 midpoint potentials for the Q_A and P^+ were -25 mV and +463 mV, respectively (Fig. 8.7a). The redox potential of Q_A is somewhat lower than found in other APB, which ranges from +5 to +150 mV (Yurkov et al., 1998a; Yurkov and Beatty, 1998), but is still considerably higher than found for anaerobic anoxygenic phototrophs (for example, *Rhodopseudomonas viridis* had a midpoint potential of -150 mV) (Yurkov et al., 1998a). Therefore, our results do not contradict those found for other APB (Yurkov et al., 1998a) and may explain the inability of ML31 to grow photosynthetically under anaerobic conditions.

In the case of $ML6^T$, measurements taken 100 ns after excitation showed the midpoint potential of Q_A/Q_A^- to be +94 mV and of the P/P^+, +415 mV. However, 50 μs after excitation the midpoint redox potential of the RC-bound cyt was +277 mV (Fig. 8.7b). Apparently, at redox potentials above +94 mV, the Q_A was oxidized before the flash and capable of accepting e^- from the P^+, but above +277 mV the RC-bound cyt was

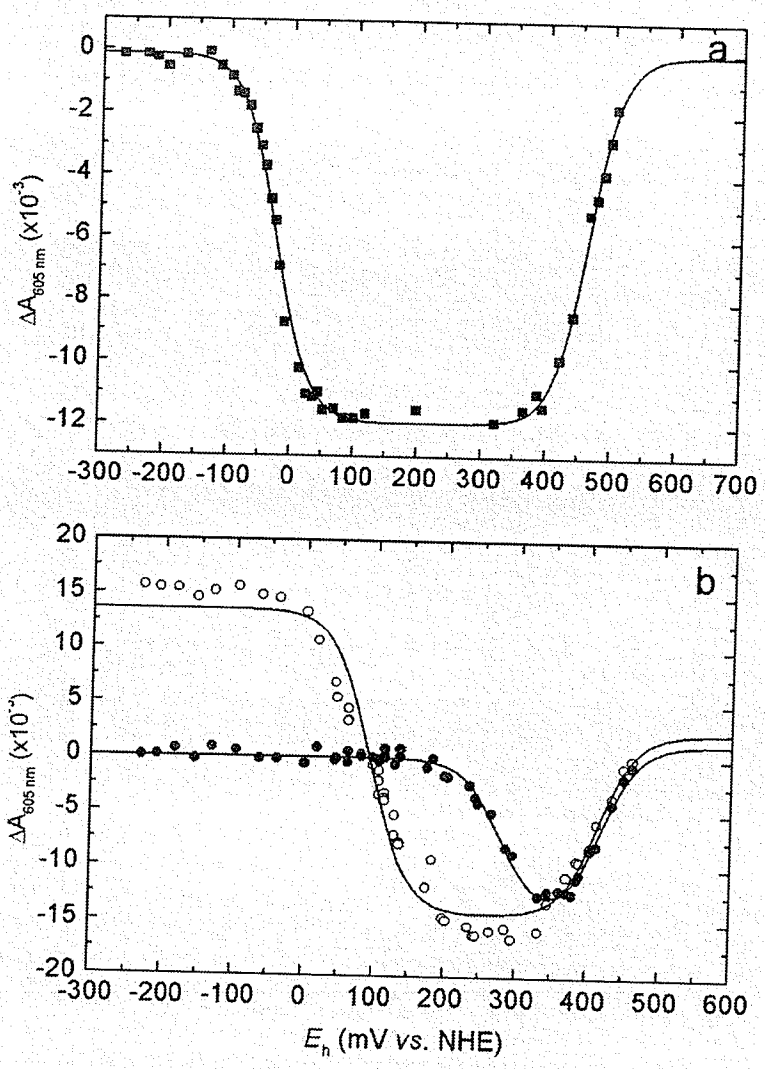

Fig. 8.7. Light induced absorbance changes measured at 605 nm, (a) 50 μs (■) after an excitation flash in membranes of ML31, and (b) 100 ns (○) and 50 μs (●) after excitation in membranes of $ML6^T$, as a function of ambient redox potential (E_h). Fully reduced conditions were achieved by addition of 10 mM $Na_2O_4S_2$, and the solutions were oxidized in increments by addition of small aliquots of 10 mM $FeK_3(CN)_6$, resulting in maximal E_h of 490 mV. The data were fitted using n=1 Nernst curves.

also oxidized before the flash and could not re-reduce P^+. Therefore, $ML6^T$ was capable of light induced e^- transfer reactions only under a very narrow range of ambient redox potentials (between +94 and +277 mV). Such peculiar limitation likely accounted for $ML6^T$'s ability to photosynthesize only semi-aerobically.

8.4.4. Photosynthetic function of carotenoids.

Carotenoid pigments are found in photosynthetic and non-photosynthetic prokayotes and eukaryotes and determine the color of organisms. In ML31 and $ML6^T$ these pigments contribute to the vibrant colors, red and purple, respectively. Carotenoids can play three major roles in photosynthesis: 1) Contribute structurally to antenna complexes. 2) Act as accessory LH pigments, allowing the use of light in the blue-green and yellow regions of the spectrum (Fraser et al., 2001) that is not absorbed by BChl. 3) Confer protection of the photosynthetic apparatus from triplet oxygen formed under illuminated aerobic conditions when excited BChl reacts with O_2. Carotenoids have been shown to quench O_2 radicals and the excited BChl triplet itself (Fraser et al., 2001).

The APB are known to produce large quantities of carotenoids relative to BChl, and in significant excess of that normally described in typical anaerobic anoxygenic phototrophs (Yurkov and Beatty, 1998). The role of these abundant carotenoids is poorly understood. To act in photoprotection they must be in direct contact with BChl in order to quench triplets effectively (Fraser et al., 2001), however most carotenoids are not associated with the photosynthetic apparatus and are distributed throughout the cell (Yurkov and Beatty, 1998; Yurkov and Csotonyi, 2006). To investigate the potential for transfer of light excitation energy from carotenoids to the RC (photosynthetic function),

fluorescence excitation measurements on whole cells, membranes and purified RC of ML31, and on whole cells, membranes and LH I-RC complexes of $ML6^T$, were performed.

Cells and membranes illuminated by light of 350-650 nm fluoresce because of the excitation energy transfer from carotenoids to BChl. Light energy that cannot be used for photosynthesis is then emitted by the BChl, between 830-890 nm. Fluorescence spectra measured for whole cells and membranes of ML31 (Fig. 8.8a) are similar in profile to the absorbance spectra of LH-RC complexes (Fig. not shown), demonstrating that only pigments associated with the complexes are involved in harvesting light, and pigments located elsewhere in the cell do not contribute energy to photosynthesis. Likewise, fluorescence spectra measured in $ML6^T$ whole cells and membranes (Fig. 8.8b) when compared to purified LH-RC (Fig 8.1b) demonstrated that extra carotenoids not incorporated into photosynthetic complexes did not collect light energy. Our results correlated with those found previously for other APB. Quantum yields of singlet energy transfer showed the majority of carotenoids did not add to light harvesting in *E. ramosum* and *R. thiosulfatophilus* (Yurkov et al., 1994). Likewise, in *E. longus* more than 70% of total carotenoids did not participate in photosynthesis (Noguchi et al., 1992). As most carotenoids are disengaged from energy transduction and evenly distributed throughout the cell, their function is uncertain. Possibly they serve as antioxidants, removing O_2 radicals of non-photosynthetic origin, or they act to filter high intensities of blue light, helping to minimize photodamage during periods of exposure to intense solar radiation (Yurkov and Beatty, 1998). Many APB are especially well endowed with polar

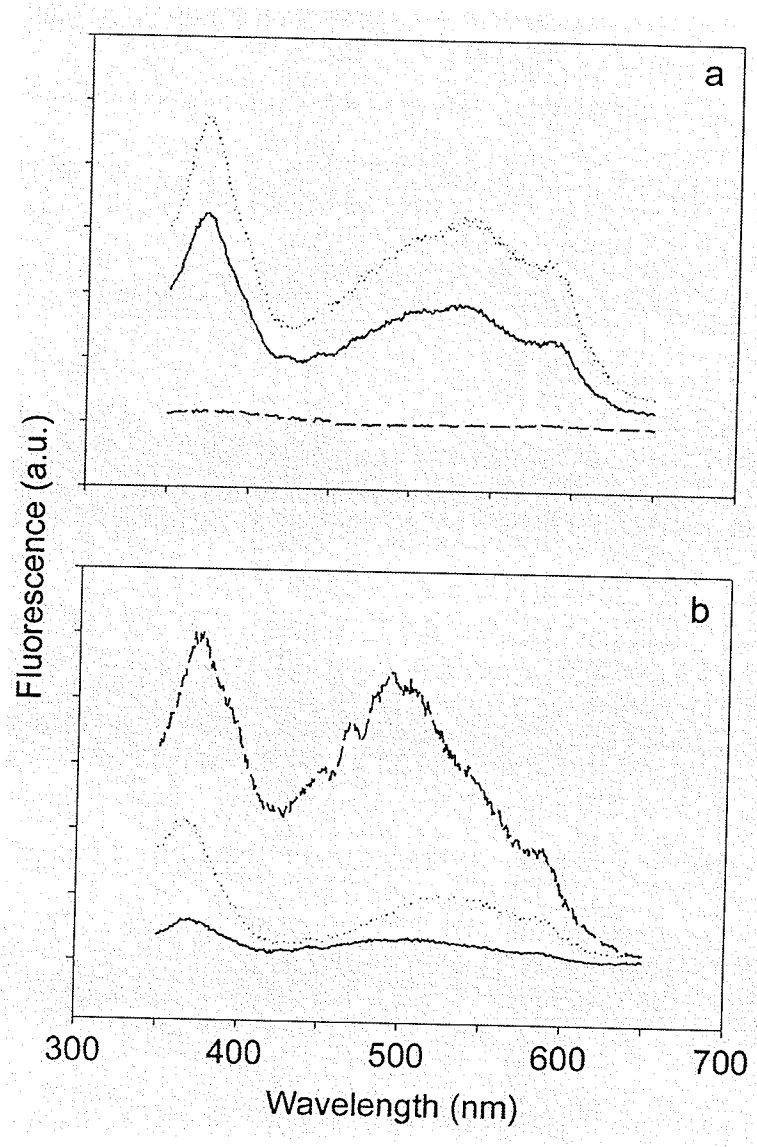

Fig. 8.8. Fluorescence excitation spectra of (a) ML31 whole cells (solid line), membrane fragments (dotted line) and purified reaction centers (dashed line), and (b) ML6^T whole cells (solid line), membrane fragments (dotted line) and purified light harvesting I-reaction center complexes (dashed line). Fluorescence was measured between 830 and 890 nm produced after excitation between 350 and 650 nm.

carotenoids (Yurkov and Csotonyi, 2003). Acquisition of the latter, which are particularly antioxidative (Krinsky, 1979) is a logical adaptation to illuminated oxic environments.

8.4.5. Electron carriers of the soluble fraction.

In all APB tested thus far the periplasmic soluble e^- carrier involved in the connection between the RC and the cyt bc_1 was a soluble cyt c_2 (Yurkov et al., 1998b), however, in anaerobic phototrophs alternative e^- carriers such as high potential iron sulfur proteins and cyt c_8 can also fulfill this function (Meyer and Cusanovich, 2003). Cyt c_2 might serve as the immediate e^- donor to P^+, as in the genera *Erythrobacter* and *Erythromicrobium*, or more frequently it serves to re-reduce a tetrahemic cyt bound to the RC.

Soluble cyts *c* have been characterized in several species. *Erythromicrobium hydrolyticum* and *S. sibiricus*, each have only one, and it is believed that this cyt functions in both respiration and photosynthesis (Yurkov and Beatty, 1998). Some other APB were shown to have 2-4 soluble cyt, and in few cases more than one have redox potentials sufficient to participate in both the respiratory and photosynthetic pathways. Unusually small cyt *c* were isolated from *R. thiosulfatophilus* (6.5 kDa and 4.0 kDa) and *E. ursincola* (6.5 kDa), but their functions have not yet been clarified. The majority of cyt isolated from APB are between 8.0 and 26.0 kDa in size. The species diversity of cyt found in phototrophs stimulated us to question if other interesting e^- carriers could be discovered in APB. Continued study of e^- transport carriers in novel strains of APB will help us to understand diversity of energetic pathways and evolution of the photosynthetic apparatus. Additionally, differences in organization of the photosynthetic apparatus have

been used as important criteria in systematics of APB (Yurkov and Beatty, 1998), and their continued investigation should help to clarify the taxonomy of this phylogentically heterogeneous group.

For this purpose we recorded reduced minus oxidized absorbance spectra and performed SDS-PAGE followed by TMBZ heme staining to calculate the molecular weights of each of the cyt present.

Reduced minus oxidized absorbance spectra recorded for the soluble fraction of ML31 revealed two soluble e^- carriers (as determined by difference peaks at 420 and 430 nm) (Fig. not shown). One was a *c* type cyt, while the other appeared to be a cyt *c'*. The molecular weights were determined to be 17 and 28 kDa (Fig. 8.9).

Redox difference spectra recorded for the $ML6^T$ soluble fraction had a high potential heme containing cyt reduced by Na-ascorbate, as well as a low potential cyt reduced only by Na-dithionite. However, TMBZ staining detected only one broad cyt band (Fig. 8.9). Due to the broad appearance of the band between around 11 and 14 kDa, it was likely that two cyt possessed nearly identical molecular weights. Based on redox titrations of the RC, the high potential heme-containing cyt could play a role in photosynthesis, because only a high potential heme would be in its reduced form at redox potentials between 270 and 400 mV that allowed the RC to function.

8.4.6. Reaction center bound tetraheme cytochrome.

Reduction of P^+ in most anoxygenic phototrophs does not proceed directly through a soluble cyt (Meyer and Cusanovich, 2003). Instead a cyt *c* intimately associated with the RC is responsible for donating e^- to P^+, and is in turn re-reduced by

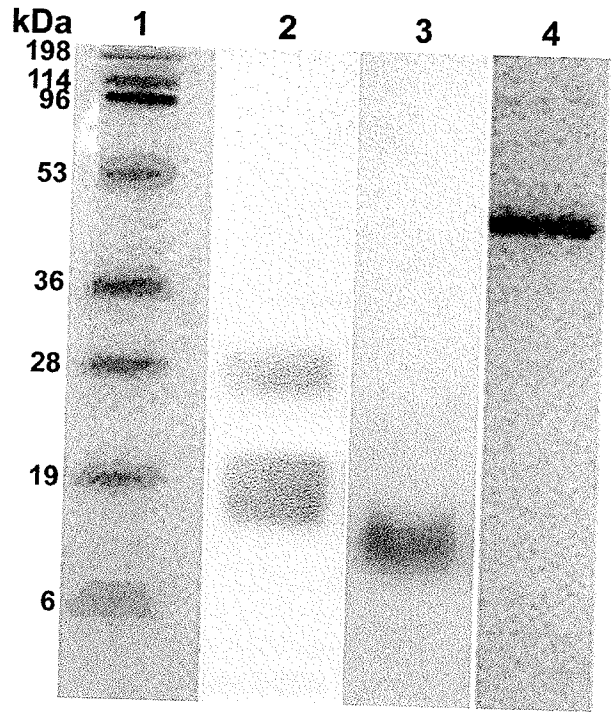

Fig. 8.9. Polyacrylamide gel electrophoresis of the soluble fractions of ML31 (lane 2) and $ML6^T$ (lane 3), and light harvesting I-reaction center complexes of $ML6^T$ (lane 4) stained with TMBZ heme stain. Standard ladder is in lane 1.

the soluble cyt. The bound cyt is generally tetrahemic, containing two high, and two low potential hemes, making the molecule fairly large. In APB the size ranges from 37.0 (*S. sibiricus*) to 44.0 kDa (*R. thiosulfatophilus*) (Yurkov and Beatty, 1998). To detect RC-bound cyt in our strains, we utilized TMBZ staining of RC polypeptides.

Reduced minus oxidized difference spectra showed the absence of a bound cyt in the RC of ML31, as was also found in light induced absorbance spectra. As expected, TMBZ staining of gels did not detect the presence of a heme containing cyt. As discussed above, light induced absorbance spectra indicated the RC bound cyt in $ML6^T$, which was also revealed by redox difference spectra and conclusively determined by TMBZ (Fig. 8.9). The cyt had a MW of about 48 kDa, which was in the range of MW reported for known RC bound cyt (Yurkov et al., 1998b).

8.5. Concluding remarks.

We have succeeded in isolating pigment protein complexes from two new species of APB isolated from the meromictic Mahoney Lake, and determined that the mild detergent Triton X-100 is suitable for releasing particularly delicate complexes from membranes, such as seen in $ML6^T$.

Photosynthesis in *P. meromictius* functions only under aerobic conditions, and the photosynthetic e^- transport involves re-reduction of the P^+ by a soluble cyt. It is interesting to note that of all APB studied thus far, RCs lacking an intimately bound cyt belong exclusively to members of the so-called *Erythrobacter-Porphyrobacter-Erythromicrobium* cluster within the α-4-*Proteobacteria*. This finding tends to disagree with suggestions that photosynthesis in APB may have come about by the action of

rampant lateral gene flow, in which entire operons have been transferred from phototrophic species to non-phototrophs. Had lateral transfer been the case, one would not expect to see homogeneity in RC organization within a phylogenetically coherent group.

Photosynthesis in *R. mahoneyensis* shows unique trends with regards to the effect of oxygen. The e^- transfer reactions were non-functional under both fully aerobic and anaerobic conditions, and only appeared to occur under conditions of reduced oxygenation. Indeed e^- transfer was only possible over a relatively narrow range of ambient redox potentials, from +270 mV (dictated by redox potential of the RC-bound cyt) and to +415 mV (P/P^+). If $ML6^T$ is truly capable of phototrophy only under microaerophilic conditions, than this strain may represent an intermediary stage in the evolution of photosynthesis, between typical anoxygenic phototrophs that grow robustly under anaerobic conditions, and typical APB, that are photosynthetically active only under full aerobiosis, supporting the idea that photosynthesis in APB has evolved to fit specific ecological niches and requirements. The peculiar trait is evidence that $ML6^T$ uses photosynthesis as an auxiliary energy source during periods of O_2 deprivation that may develop when other rapidly respiring heterotrophs within the community consume available O_2. Because Mahoney Lake is a relatively eutrophic habitat, such a situation can be encountered.

8.6. Aknowledgements.

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Chapter 9.

Final comments and future perspectives.

9.1 Major findings of the thesis.

The first major goal of this study was to examine several new isolates of phototrophic and metalloid resistant bacteria from two different extreme environments, the meromictic Mahoney Lake, and deep ocean hydrothermal vents of the Pacific Ocean.

Aim 1. (See section 1.5.1.) The phototrophic strains from Mahoney Lake exhibited a plethora of morphological forms (Fig. 2.1-2.6) and several had absorbance characteristics indicating a new photosynthetic apparatus organization (Fig. 2.7, 2.8). Physiologically, most were revealed as new APB, although two strains of purple non-sulfur bacteria were also characterized. Several used a variety of organic substrates similar to other APB (Yurkov and Beatty, 1998a), whereas others were quite fastidious, surviving on relatively few substrates, and having growth factor requirements (Table 2.3). The strains were highly tolerant to extremes of pH and salinity (Table 2.3, 2.4, Fig. 2.9) as expected of bacteria living in an alkaline meromictic lake. Phylogenetically they belonged to the *α-Proteobacteria*, intermingled with both phototrophic and non-phototrophic relatives (Table 2.5).

Aim 2. (See section 1.5.2.) The heavy metalloid resistant bacteria recovered from hydrothermal vents of the Juan de Fuca Ridge formed black colonies when grown with K_2TeO_3 and were orange to red in the presence of Na_2SeO_3 (Fig. 3.1). Microscopy revealed that addition of K_2TeO_3 and Na_2SeO_3 provoked changes in cell morphology together with accumulations of light refractile and e^- dense globules, inside and outside the cells (Fig. 3.2, 3.3). All isolates resisted and reduced extremely high levels of both TeO_3^{2-} and SeO_3^{2-} regardless of the metalloid used for their initial isolation (Table 3.2). Physiologically they were obligately aerobic chemoorganoheterotrophs, using a wide

variety of substrates (Table 3.2) and were tolerant to fluctuations in pH, salinity and temperature, which might be an adaptation to their habitat where hot, low-pH vent fluids mix with colder, more alkaline surrounding water (Feely et al., 1987). Four of the strains were closely related to species of *Pseudoalteromonas* (Fig. 3.4), although members of this genus have not previously been shown to resist and reduce oxyanions of Te and Se.

Aim 3. (See section 1.5.3.)Strains from hydrotherms of the Juan de Fuca Ridge and the meromictic Mahoney Lake have thus far yielded two new species of the genus *Pseudoalteromonas*, *P. spiralis* and *P. telluritireducens* (Rathgeber et al., 2006) and one species of a novel genus, *Roseicyclus mahoneyensis* (Rathgeber et al., 2005). Additionally a new species, *Porphyrobacter meromictius*, has been proposed. Taxonomic identification of strains serves to organize our knowledge of complex microbial relationships using a coherent and useful system, and makes this information and microorganisms available to the scientific community.

Aim 4. (See section 1.5.4.) Major questions were raised following the description of the extremotolerant APB *Citromicrobium bathyomarinum*, the first phototrophic organism cultured from the permanently dark deep ocean (Yurkov and Beatty, 1998b; Yurkov et al., 1999). Is *C. bathyomarinum* endemic to hydrothermal vent plumes, or does it live throughout the water column? Is its photosynthetic apparatus functional, in terms of light induced e^- transfer, and can it be used to supply an auxiliary source of energy? Therefore, the second major goal of the thesis was to answer these questions, first by searching for cultivable phototrophs throughout the water column at five sites in the eastern Pacific Ocean (Table 7.1), comparing new isolates to *C. bathyomarinum*, and then

examining the photosynthetic apparatus composition. Lastly the ability to carry out light induced e^- transfer was analyzed.

The search for APB in the Pacific Ocean yielded 13 yellow BChl *a*-containing strains from the surface to 2379 m depths (Table 7.2), which were spectrally, morphologically and physiologically similar to *C. bathyomarinum* (Fig. 7.1, 7.2; Table 7.3). However, strain C23 obtained from the surface had different pigment content, and lower tolerance to salinity (Table 7.3) than those recovered from the deep ocean. Phylogeny confirmed the identity of all deep ocean isolates as strains of *C. bathyomarinum*, whereas C23 was genetically distant, related most closely to the genus *Erythrobacter* (Fig. 7.3). Our results confirm that *C. bathyomarinum* can be repeatedly cultured from depths below 500 m and may be endemic to the deep ocean.

To confirm the organization of the photosynthetic apparatus in *C. bathyomarinum*, photosynthetic units from strains JF-1 and C8 were isolated and revealed the presence of a LH I complex with a major absorbance peak at 867 nm and a photosynthetic RC at 801 nm (Fig. 7.1). No evidence of a LH II complex was found. To gauge its ability to carry out photosynthetic e^- transport and thus produce cellular energy from light, photochemistry was investigated showing that cells were photosynthetically competent under aerobic conditions, although e^- transfer between the RC and its bound cyt was slow (Fig. 7.4). Redox titrations of the P and Q_A revealed that photosynthesis was only active under relatively oxidized conditions and the midpoint potential of Q_A was likely responsible for *C. bathyomarinum*'s inability to grow phototrophically in the absence of O_2 (Fig. 7.5). Consequently, *C. bathyomarinum* is capable of light driven e^-

transport and should therefore be able to satisfy a portion of its energetic requirements when light is present.

Aim 5. (See section 1.5.5.) The final objective was to examine the organization of the photosynthetic apparatus in the Mahoney Lake species *R. mahoneyensis* and *P. meromictius* to better understand the peculiarities of light induced e^- flow in APB, to determine if photosynthesis is governed by the redox potential of Q_A as observed in some other species (Yurkov et al., 1998), and to search for APB that behave differently with respect to the effect of O_2 on photosynthesis.

Photosynthesis in *P. meromictius* functions only under aerobic conditions, and photosynthetic e^- transport involves re-reduction of the P^+ by a soluble cyt (Fig. 8.2). It is interesting that of all APB studied thus far, RCs lacking an intimately bound cyt belong exclusively to members of the so-called *Erythrobacter-Porphyrobacter-Erythromicrobium* cluster within the α-4-*Proteobacteria*. *R. mahoneyensis* shows unique behavior with regards to the effect of oxygen. The e^- transfer reactions were non-functional under both fully aerobic and anaerobic conditions, and only appeared to occur under conditions of reduced oxygenation (Fig. 8.4, 8.5). Indeed e^- transfer was only possible over a relatively narrow range of ambient redox potentials, from +270 mV (dictated by redox potential of Q_A) and to +415 mV (RC-bound cyt) (Fig. 8.7). This suggests the first finding of an APB capable of phototrophy only under microaerophilic conditions, and may represent an intermediary stage in the evolution of phototrophic bacteria.

9.2. Future perspectives.

The discoveries in this work have prompted several new questions, and opened the door for new investigations.

1. Why do many APB from Mahoney Lake exhibit nutritional restrictions, not commonly observed in other species of this group? It will be important to determine the specific growth factors required by fastidious strains. Once these factors are known, it will be interesting to determine why such requirement is common among Mahoney Lake APB.

2. In light of high resistance to TeO_3^{2-} reported for several APB (Yurkov et al., 1996), it will be interesting to determine if the Mahoney Lake isolates are capable of metalloid reductions. Considering the tolerance to other environmental stresses, it is possible that they will be able to resist and reduce high concentrations of toxic oxyanions. Physiological tests such as those described in Chapter 3 could be used to evaluate this potential.

3. The mechanisms of TeO_3^{2-} and SeO_3^{2-} reduction need to be investigated. Most past research has focused on strains with significantly lower minimum inhibitory concentrations than those discovered at the Juan de Fuca Ridge. Given that several phylogenetically distant species capable of reducing high levels of metalloids are now available (Yurkov and Csotonyi, 2003; Rathgeber et al., 2006) it should be established whether mechanisms of reduction differ in these organisms from those

previously reported. This will involve construction of mutants, determination of genetic loci and gene products responsible for metalloid resistance and kinetic studies of the enzymes. Once the biochemical and molecular mechanisms allowing such high level of reduction are understood, model organisms can be chosen for large-scale bioremediation experiments.

4. With the discovery of strains aerobically reducing high levels of TeO_3^{2-} and SeO_3^{2-}, interest has surged in heavy metal and metalloid resistant organisms in the vicinity of deep ocean hydrothermal vents. Since our report, vent communities have yielded strains capable of dissimilatory metalloid reductions (anaerobic Te, Se and V respiration) (Csotonyi et al., 2006) and bacteria resisting high levels of mercury (Vetriani et al., 2005). Truly these sites have great potential as a source for organisms with multiple metal resistance.

5. The discovery that *C. bathyomarinum* is distributed throughout the lightless water column in the Pacific Ocean leads to a conundrum: What is its ecological role? Is the potential for photosynthesis an advantage to an organism that lives in a permanently dark environment? To answer these questions it is important to know if the photosynthetic components are actually expressed by cells growing in the dark deep ocean. Studies involving *in situ* reverse transcription of *puf* genes from concentrated deep ocean samples may help shed light on this problem.

6. The taxonomic situation in the α-*Proteobacteria* needs to be clarified. Existing genera can often not be differentiated by 16S rRNA gene phylogenies, and new species are routinely included into dissimilar taxa based entirely on 16S sequence similarity (Rathgeber et al., 2004; Yurkov and Csotonyi, 2006). Phylogenetic analyses of housekeeping genes should help to resolve the confusion caused by the inclusion of non-BChl synthesizing species into phototrophic genera, and should help to realize which characters will remain important in modern taxonomy.

7. Several strains of APB from Mahoney Lake and the Pacific Ocean may represent new genera/species and still require taxonomic characterization. Identification, based on further phylogenetic analysis and phenotypic properties, such as performed in Chapters 4-6, will help clarify the diversity of this group and will bring scientific interest to novel species.

8. Although similar to typical AnAP, photosynthesis in APB occurs only under oxidized conditions. Several biochemical reasons have been proposed to explain such behavior including redox potential of the Q_A (Yurkov et al., 1998; Chapter 8) and the redox state of RC bound tetraheme cyt (Schwarze et al., 2000). However, it is still not clear how this difference is encoded genetically. An in depth study of the APB photosynthetic superoperon will be necessary to provide an answer.

9. The discovery that *R. mahoneyensis*, ML6 is capable of photosynthesis only within a very narrow range of ambient redox potentials leads to the conclusion that the

organism supplements its energy requirements with light under microaerophilic conditions. To test this hypothesis, it will be important to determine how O_2 affects BChl synthesis. Chemostat experiments may show how light availability impacts cellular energy budgets under variable O_2.

9.3. References.

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