Anoxygenic Photosynthetic Communities and Heavy Element Transformations in Extreme

Environments: Hydrothermal and Hypersaline Ecosystems

By

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Abstract.

The current research project investigated the anoxygenic phototrophic and metal(loid) transforming bacteria of hypersaline and deep ocean hydrothermal environments. The East German Creek brine springs, an unusual flowing hypersaline system, was enumerated using classical techniques. Subterranean sulfide supported purple sulfur and nonsulfur bacteria, but at the highly oxygenated surface, aerobic anoxygenic phototrophs (AAP) were numerically dominant (up to 16-36% of cultivable bacteria). Strains (EG8, EG13, EG17, EG19) with unusual phylogenetic affiliation and novel photosynthetic and metal(loid) reducing traits were described taxonomically. *Chromocurvus halotolerans* gen. nov., sp. nov. was proposed as a second example of a gammaproteobacterial AAP. It exhibited bent rod-shaped cells, unusual among AAP. Facultatively anaerobic *Charonomicrobium ambiphototrophicum* gen. nov., sp. nov. was capable of both aerobic and anaerobic anoxygenic photosynthesis, and incapable of photoautotrophy, distinguishing it from both AAP and purple nonsulfur bacteria. *Roseovarius vanadiphilum* sp. nov. surprisingly produced 4.5 times more biomass and 2 times more bacteriochlorophyll (BChl) at extremely high $NaVO_3$ concentration (7.5 g/l) than in metal-free medium.

A second novel metabolic mode, anaerobic respiration on the toxic metalloid tellurate, was described for a relative of non-phototrophic *Shewanella frigidimarina* (ER-Te-48), from deep ocean hydrothermal vent *Paralvinella* worms at Explorer Ridge in the Pacific Ocean. Other strains respired on SeO_3^{2-} (ER-Se-17L), VO_3^- (ER-V-6), and VO_4^{3-} (AV-V-25). These organisms provided the first examples of anaerobic respiration on Te, Se and V at hydrothermal vents.

High level resistance of AAP to metal(loid)s prompted investigation of the influence of TeO_3^{2-} on photosynthetic pigment production in species including *Erythromicrobium ramosum* (from a terrestrial hydrothermal system) and *Erythrobacter litoralis* (from a hypersaline supralittoral system). Tellurite enhanced photosynthetic pigment production up to 3.4 times, consistent with an antioxidant carotenoid-based defense mechanism. However, in *E. litoralis* BChl precursors such as Mg protoporphyrin or its monomethyl ester also accumulated, indicating biosynthetic pathway interruption.

In hydrothermal and hypersaline ecosystems, largely devoid of eukaryotic phototrophs but often enriched in metal(loid)s, AAP and metal(loid) reducers are key modulators of nutrient and toxin availability. The presented results on their ecology, physiology and biochemistry have important implications for theoretical understanding of extreme environments and hold potential for biotechnological applications.

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List of abbreviations.

AAP   aerobic anoxygenic phototrophs

ATP   adenosine triphosphate

BChl   bacteriochlorophyll

BPhe   bacteriopheophytin

CCCP   carbonyl cyanide *m*-chlorophenylhydrazone

CFU   colony forming unit

Chl   chlorophyll

EGC   East German Creek

GNSB   green nonsulfur bacteria

LH   light harvesting

λ_{max} peak absorbance wavelength of a pigment

MgP   Mg protoporphyrin

MgPME   Mg protoporphyrin monomethyl ester

MIC   minimum inhibitory concentration

NSERC   Natural Science and Engineering Research Council

PCR   polymerase chain reaction

PNSB   purple nonsulfur bacteria

ppm   parts per million

ppT   parts per thousand

PS   photosystem

Q_A primary photosynthetic electron acceptor

RC	reaction center
RO	rich organic
R/V	research vessel
TDS	total dissolved solids
TLC	thin layer chromatography

Chapter 1.

Introduction.

1.1. Introduction to extreme environments

1.1.1. Definition of extreme environments and extremophiles

Extreme environments represent the fringes of the biosphere. Their physical and chemical parameters fall near upper and lower limits that biological systems can tolerate. Brock (1979) described extreme environments as those in which "species diversity is low, and some taxonomic groups are missing". Only life forms possessing extensive adaptations through natural selection survive under these conditions. Organisms are 'extremophilic' if maximal fitness requires extreme conditions, or 'extremotolerant' if they survive extremes but perform best under mesic conditions. Extremophiles are further classified according to the extremes they tolerate as thermophiles (high temperature), psychrophiles (low temperature), halophiles (high salt concentration), acidophiles (low pH), alkaliphiles (high pH), barophiles (high pressure), xerophiles (desiccation) and osmophiles (low water activity) (Rothschild and Mancinelli, 2001). The term 'metallophile' was coined for species preferring high, normally toxic metal concentrations (Nies, 2000). Microbiological investigation of extreme environments is quickly rising into the limelight with the advent of new sampling techniques and increased accessibility (Fenchel *et al.*, 1998). Studies have demonstrated that despite the restricted distribution of many extreme environments, they are disproportionately valuable to research. Such habitats yield a plethora of microorganisms from broad phylogenetic clades with theoretical significance to biodiversity, evolution of metabolic pathways, origins of life and astrobiology. These microbes also possess immense biotechnological potential, especially for bioremediation and biometallurgy (Zannoni *et al.*, 2008).

1.1.2. Diversity of extreme environments

Certain extreme environments are uncommon on earth today. Hyperthermal reducing conditions rich in dissolved metals characterized early earth (Lovley, 2002). Today, thermophiles are mainly restricted to geologically active areas such as terrestrial and deep-ocean hydrothermal vents. These habitats truly straddle the interface between habitability and intolerability: in the deep ocean, a few cm or mm may separate 2^oC ocean water above from >300^oC fluids beneath (Kelley *et al.*, 2002). Hydrothermal vents are the oceanic equivalent of geysers, and result when water percolating through the subsurface at volcanically active oceanic spreading centers is magmatically superheated (up to 400^oC), acidified (pH as low as 3.3) and loaded with H_2S and various toxic metals leached from basalt prior to its release into the ocean (Kelley *et al.*, 2002). Rapid cooling upon mixing with ocean water causes dissolved minerals to precipitate into smoke-like particles, leading to the term "black smoker chimneys" to describe the spires of metal-sulfide that result from deposition (Fig. 1.1A). The effluent rises until it reaches neutral buoyancy, forming a plume. Hence, hydrothermal vent fluids and associated mineral deposits present microorganisms with several different extremes: high pressure, temperature, acidity and toxic metal concentrations. Vents support abundant biological communities (Fig. 1.1B) because they are rich in chemical species sufficiently reduced to drive energetically expensive biochemical reactions (Kelley *et al.*, 2002). This biotope is still incompletely mapped because of the logistic difficulty of deep ocean surveys, but it is restricted to faults and spreading centers, such as the Juan de Fuca Ridge and Explorer Ridge in the Eastern Pacific (Delaney *et al.*, 1992).

Other extreme environments are widespread: hyperbaric conditions dominate extensive marine areas, and cold temperatures limit life near both poles. Hypersaline habitats occur wherever marine waters desiccate, as on intertidal flats (Yurkov and van Gemerden, 1993a), but
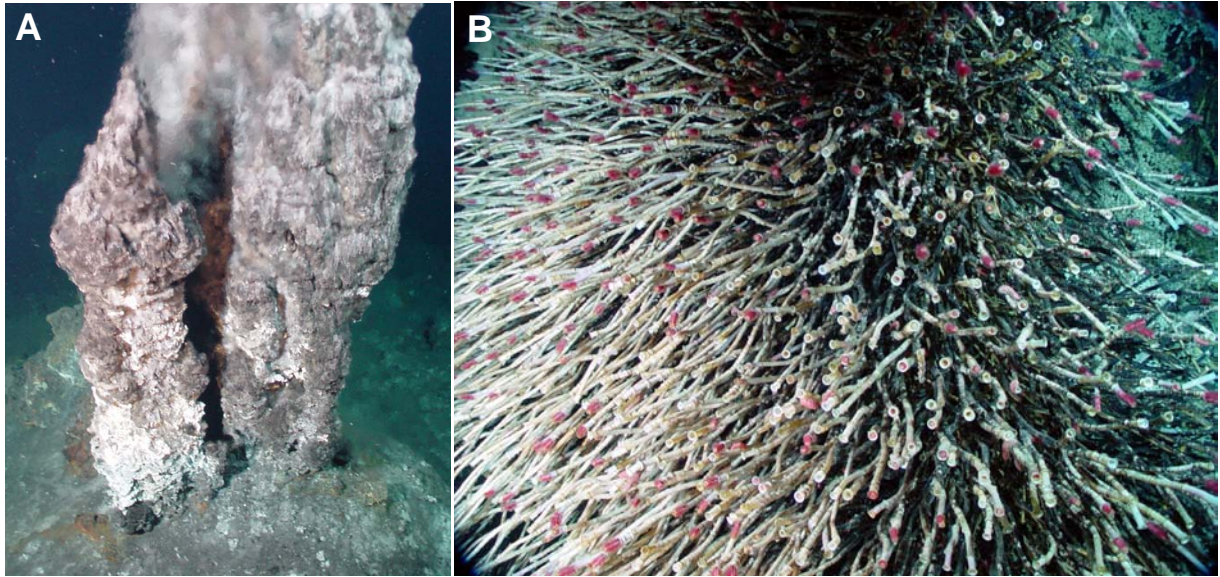
Fig. 1.1. Photographs of deep ocean hydrothermal vents at Explorer Ridge in the Pacific Ocean.
(A) A black smoker chimney located in the Magic Mountain area emitting high temperature vent
fluids. **(B)** Thick colony of tubeworms (*Ridgeia piscesae*) growing on the cool temperature
sulfide chimney named "Zooarium" after its abundant and diverse biota. Source of photos:
National Oceanic and Atmospheric Administration

their distribution is not constrained by the current extent of ocean. Salt-rich ancient marine sediments now exposed at the surface may enrich overlying lakes, sometimes causing them to accumulate sufficient solutes at depth to resist turnover of the hypolimnion, in which case they are termed meromictic lakes (Javor, 1989). Examples include Great Salt Lake in the United States, the Dead Sea in Israel and Mahoney Lake in Canada (Javor, 1989; Yurkova et al., 2002). Similarly, ground water passing through marine geological horizons may generate hypersaline springs. Compared to meromictic lakes and intertidal zones, hypersaline springs are relatively rare, because the conditions that need to be met for their existence are rather restrictive: sufficient hydrostatic pressure to force free-flowing subterranean water to the surface after passing through enough salt-rich strata to appreciably load it with solutes. A recently discovered hydrological system near Lake Winnipegosis in Manitoba (Fig. 1.2) meets the requirements for generating hypersaline springs (McKillop et al., 1992). Water that was forced into Devonian marine sediments of the Western Canadian Sedimentary Basin by the hydrostatic head of glaciers during the Wisconsian ice age now exits at a series of seeps and springs of varying salinity dominated by NaCl (Grasby et al., 2000). A locality known as East German Creek (EGC) has a total dissolved solids content of 56.7‰ to 67.3‰ (Csotonyi et al., 2008). A high flow rate of 4800 l/h generates a series of runoff streams lined by salt precipitates, and orange iron oxyhydroxide-rich minerals (McKillop et al., 1992) and microbial mats (Csotonyi et al., 2008) (Fig. 1.2). This unusual ecosystem combines the steep salinity and chemical gradients characteristic of meromictic lakes with the flowing water of rivers.

Certain extreme environments increase due to human activity. Xeric conditions abound on large tracts of arid soil, which will likely expand under the influence of global climate change (Belnap et al., 2004). Metal mining and ore processing increasingly generate metalliferous and
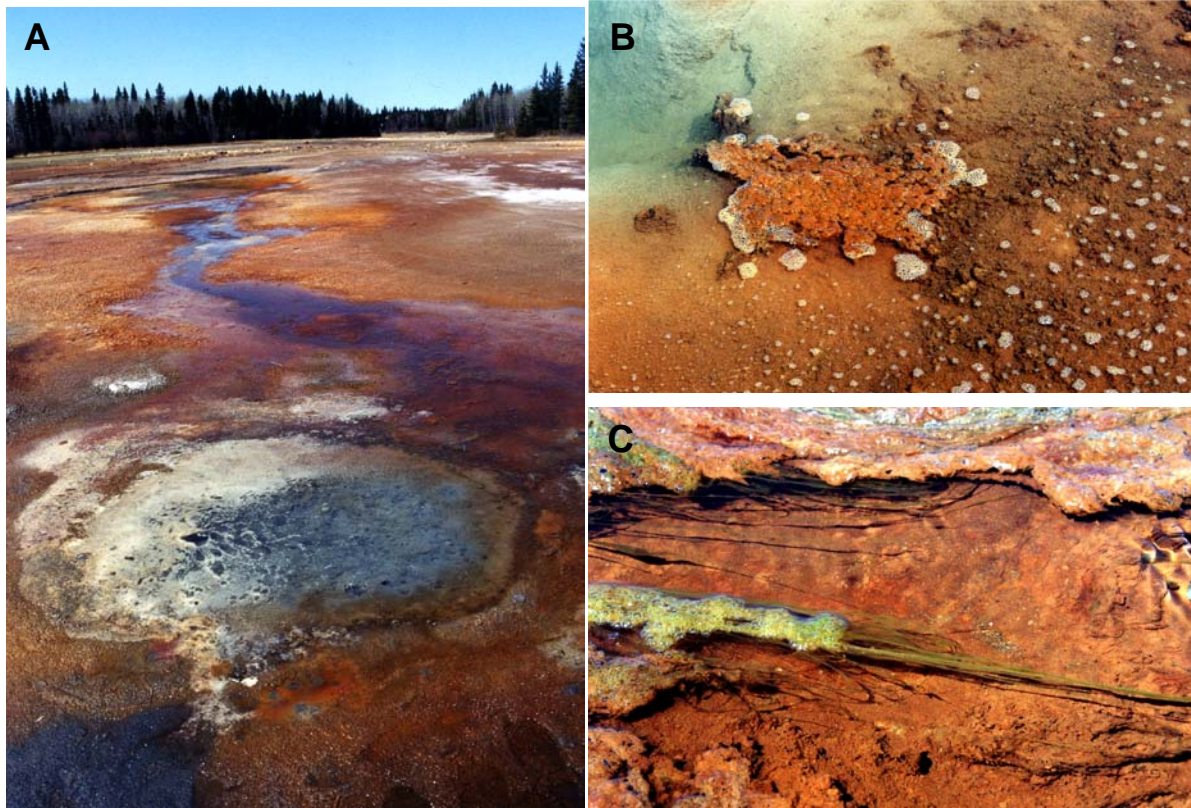
Fig. 1.2. Photographs of the hypersaline East German Creek springs. **(A)** Spring pool and associated effluent stream. The orange hues are due to iron deposits and the white patches in the background are NaCl evaporates. Foam at the water surface is due to gas emissions from the spring. **(B)** Close-up of floating microbial communities that develop around the margin of spring pools. **(C)** Close-up of microbial communities in effluent stream, showing streamers of algae and iron-impregnated floating mat-like growth.

acidic effluent waters (Nies *et al*., 1999). The hydrology of some natural extreme habitats such as hydrothermal vents and hypersaline systems also elevates concentrations of metals, such as vanadium (V), or metalloids over the background (Kelley *et al*., 2002; Ong *et al*., 1997). Metalloids are elements that share some properties with both metals and nonmetals, occupying intermediate positions on the periodic table. They include boron (B), silicon (Si), germanium (Ge), arsenic (As), selenium (Se), antimony (Sb), tellurium (Te) and polonium (Po) (Chasteen and Bentley, 2003; Zannoni *et al*., 2008). In their soluble forms, some metalloids are very toxic to prokaryotes and eukaryotes (Zannoni *et al*., 2008). For example, Te belongs to the Group III elements, classified as nonessential and toxic to most bacteria (Borsetti *et al*., 2009). We refer to metals and metalloids collectively as metal(loid)s.

Extreme habitats harbor exceptional organisms. Some of the most metal(loid) resistant microorganisms hail from hydrothermal systems (Rathgeber *et al*., 2002; Yurkov *et al*., 1996), and hypersaline evaporation ponds (de Souza *et al*., 2001). These organisms are explored in Sections 1.5. and 1.6. Furthermore, the exposure of many plant-deficient terrestrial thermal and hypersaline environments to solar radiation also promotes the development of diverse microbial communities dominated by phototrophs. The following Section 1.2. introduces anoxygenic photosynthesis as a necessary background for a deeper discussion of obligately aerobic anoxygenic phototrophs in Section 1.3., and in extreme environments in Section 1.4. Anoxygenic phototrophs are also the most highly metal(loid) resistant bacteria known, and these are covered in Section 1.7., in the context of the interaction of metal(loid) resistance and photosynthetic function and pigment synthesis.

1.2. Anoxygenic versus oxygenic photosynthesis

Of the prokaryotic taxa in extreme environments, phototrophs are pivotal because they are the main primary producers and important light-assisted secondary consumers in illuminated extreme habitats that exclude most eukaryotic photosynthesizing organisms. Photosynthesis, the biological transduction of light to cellular energy, is accomplished by photosystems (PS) composed of tightly regulated electrochemical concerts of special pigment molecules (chlorophylls, bacteriochlorophylls and carotenoids), and a variety of electron shuttling compounds including wavelength modulating protein complexes within which pigments are incorporated, iron-sulfur proteins, cytochrome complexes and quinones (Madigan *et al*., 2003).

Photosynthesis exists in two forms. Oxygenic photosynthesis, found in cyanobacteria, algae and plants, is so named because the chlorophyll(Chl)-mediated photolysis of water yields O_2 as a by-product of the generation of reducing power for noncyclic electron flow (Fig. 1.3A) (Madigan *et al*., 2003). Light quanta strike a Chl molecule (P680) in the PSII RC, exciting it and initiating the funneling of an electron down a transport chain. During an exergonic stepwise passage through pheophytin, plastoquinone, cytochromes and plastocyanin, its energy is conserved via transmembrane proton extrusion followed by ADP phosphorilation. The electron then reduces the Chl (P700) of the PSI RC, which following excitement by a second photon, donates the electron to iron sulfur proteins, ferredoxin and finally $NADP^+$ reductase, generating reducing power in the form of NADPH (Fork and Herbert, 1993). Because the electron is not returned to the PSII RC, this form of photosynthesis is known as noncyclic photophosphorilation. The O_2 evolved initially oxygenated the atmosphere, and selected for high aerotolerance of oxygenic phototrophs.
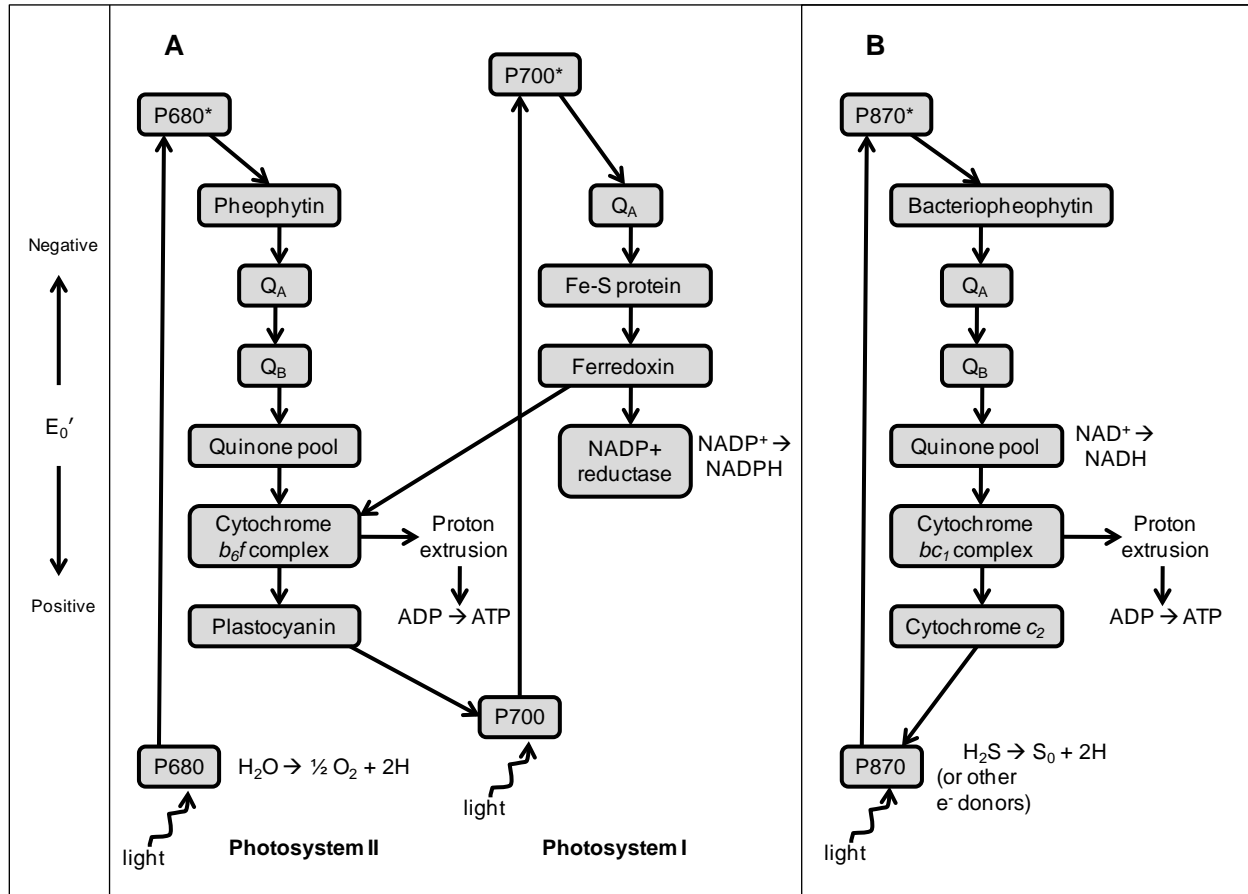
Fig. 1.3. Schematic representation of **(A)** oxygenic and **(B)** anoxygenic photosynthesis. Electron flow (straight arrows) through major photosynthetic apparatus components (boxes) is depicted, arranged vertically according to their approximate relative redox potentials (E_0'). Also shown are sites of incident light absorption, electron donor oxidation, energy generation (via transmembrane proton extrusion followed by ADP phosphorylation) and generation of cellular reducing power. Based on Madigan *et al*. (2003).

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Anoxygenic photosynthesis is an ancestral, usually anaerobic, metabolic mode of energy

generation in *Proteobacteria* (purple sulfur and nonsulfur bacteria), *Firmicutes* (heliobacteria),

Chlorobi (green sulfur bacteria) and *Chloroflexi* (green nonsulfur bacteria) (Madigan *et al*.,

2003). The pigment bacteriochlorophyll (BChl) instead of Chl facilitates energy conservation by

cyclic photophosphorylation involving a single PS only (Fig. 1.3B). The configuration of the

photosynthetic electron transport chain depends on the anoxygenic photosynthetic group, but

Proteobacteria serve as suitable representatives of its function. In this process, the energy of

photons is transferred through transmembrane light harvesting (LH) I and II complexes

(accessory antennae containing BChl surrounded by carotenoids that widen the available range

of wavelengths and intensities of captured light) to the RC complex (containing a special pair of

BChl and bacteriopheophytin). From here, an energized electron is channeled through

ubiquinones (Q_A and Q_B), a membrane lipid phase quinone pool, the transmembrane cytochrome

bc_1 complex (where the energy of the electron is harnessed to transocate two protons across the

membrane, generating the electrochemical gradient responsible for regenerating ATP), and

finally back to the RC via soluble and/or RC-bound cytochromes *c*, depending on the species

(Jones, 2009). Use of H_2 or reduced S or C compounds as electron donors precludes O_2 evolution

(Madigan *et al*., 2003). Because S^{2-} and H_2 are only stable in the absence of oxidizing agents,

anoxygenic photosynthesis originally developed for strictly anaerobic operation on a reducing

early earth.

In today's highly oxygenated biosphere, most anoxygenic phototrophs are restricted to

microhabitats providing both light and these specialized sources of reducing power, unlike

oxygenic phototrophs, which use abundantly available water as a source of electrons.

Anoxygenic phototrophs often form well-structured communities in laminated mats, the

component organisms distributed according to O_2 sensitivity and preferred radiation wavelength range (Caumette *et al*. 1999). In the deepest layers live green sulfur bacteria, the most O_2 sensitive and sulfide requiring organisms. Above them may exist a layer of purple sulfur bacteria capped by oxygenic phototrophs (Ward *et al*., 1998). Interspersed and overlapping with the aerobic and purple sulfur layers may be purple nonsulfur and green nonsulfur bacteria (Caumette *et al*. 1999). The latter two groups are facultatively aerobic heterotrophs, and can also photosynthesize, but only anaerobically. Compared to Chl *a* in oxygenic phototrophs, (*in vivo* λ_{max} = 680 nm; red light), BChl absorbs at longer wavelengths: ~800 nm to 1020 nm (near infrared), depending on the structural variant of the pigment and its protein environment. Anoxygenic phototrophs utilize intervals of the electromagnetic spectrum complementary to the light removed by overlying algae and cyanobacteria (Caumette *et al*. 1999). Not all groups are present in all communities, and the physical structure of the assemblage can vary substantially. For example, purple sulfur bacteria populate the water column of some meromictic lakes in a stratum known as a "bacterial plate" (Overmann *et al*., 1994) while green sulfur bacteria are dispersed throughout deep anoxic waters of the Black sea (Koblížek *et al*., 2006).

1.3. Aerobic anoxygenic phototrophs: a paradigm shift

Three decades ago, the unexpected discovery of obligately aerobic bacteria, in which synthesis and function of BChl *a* actually requires O_2, heralded a paradigm shift in photosynthetic microbiology: that anoxygenic photosynthesis is not exclusively an anaerobic process (Shiba *et al*., 1979). Although they are often associated with oxygenic phototrophs at the surface of laminated mats (Yurkov and van Gemerden, 1993a), the aerophilic nature of aerobic anoxygenic phototrophs (AAP) does not limit them to these mats. They occur throughout the

oxic biosphere, in nearly every environment sampled (Yurkov and Csotonyi, 2009). AAP phylogeny reveals close affinity with purple phototrophs, but stark differences exist in regulation and function of the photosynthetic apparatus. The AAP are currently gaining increasing research interest, not only because their abundance has implications to global carbon cycling but because exceptional resistance to toxic metal(loid)s grants them great potential applicability to biotechnology. This is especially true of AAP from extreme environments, which frequently tolerate multiple industrially relevant extremes. AAP possess the highest reported bacterial resistance to toxic oxyanions of Te. Studies of metal(loid) reducers and phototrophs, especially AAP, from extreme environments, as well as the interactions between photosynthesis and metal(loid) resistance, are likely to be productive on both theoretical and applied fronts. It is this waxing interest that centralizes the AAP within the scope of the current project.

1.3.1 Taxonomy, phylogeny and evolution of AAP

The AAP are mainly *Alphaproteobacteria*, with one published species each from the *Betaproteobacteria* (*Roseateles depolymerans*) (Suyama *et al*., 1999) and *Gammaproteobacteria* (*Congregibacter litoralis*) (Spring *et al*., 2009). Genomic analyses of photosynthetic operon mRNA from aerobic ocean and river water samples (Béjà *et al*., 2002; Waidner and Kirchman, 2005) and recent enumeration of novel extreme environments (Csotonyi *et al*., 2008, Jiang *et al*., 2009) indicate that many new alpha-, beta- and gammaproteobacterial members await description. The past two decades have witnessed an explosion in the discovery of new AAP. From one genus (*Erythrobacter*) prior to 1991 (Shiba, 1989), the number of described AAP have grown to 57 species in 35 genera (Yurkov and Csotonyi, 2009) (Table 1; Fig. 1.4).
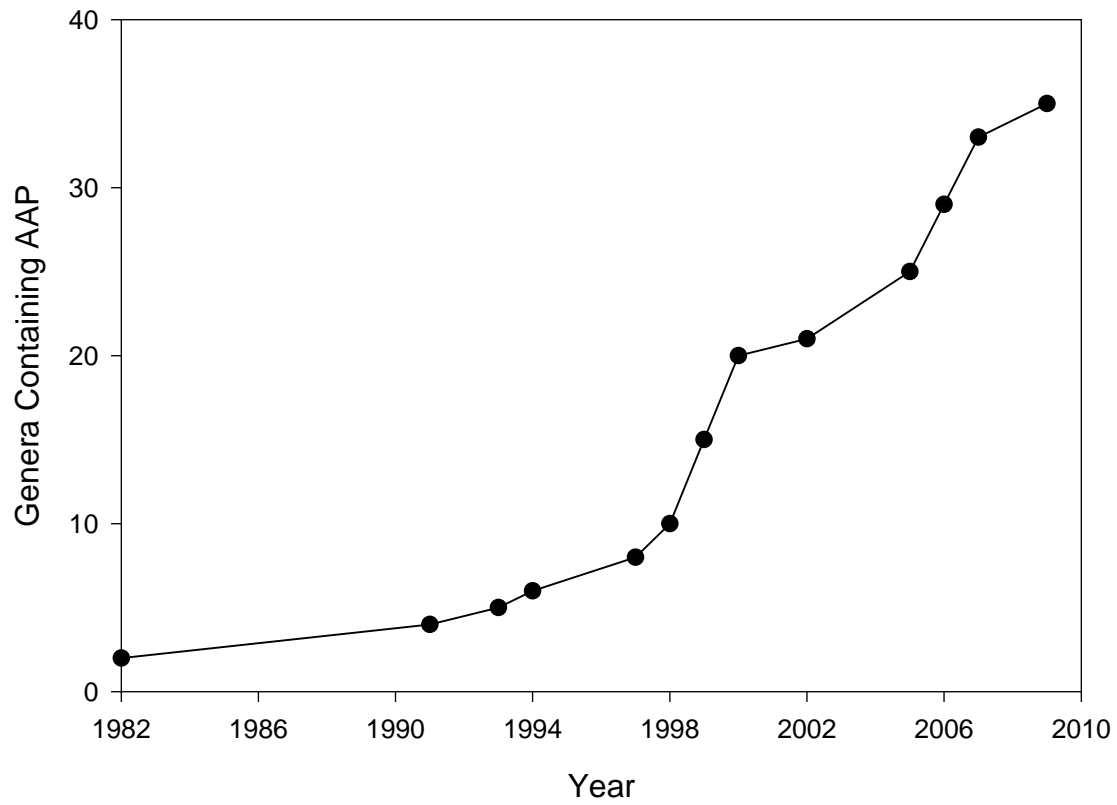
Fig. 1.4. Taxonomic richness of AAP. Cumulative growth in number of genera to which AAP

have been assigned over 27 years. Source: Table 1.

Table 1. Phenotypic and physiological characteristics of AAP.

Species and Reference	Morphology and color	in vivo BChl peaks (nm)	Temp (°C)	pH	Salinity (%)	Native Habitat
			Optimum Growth Conditions			
Acidiphilium acidophilum (**Hiraishi** *et al*., **1998**)	**Rods; White to cream**	**NA** [1]	**25-30**	**3-3.5**	**NA**	**Acidic mine drainage**
Acidiphilium angustum (**Wichlacz** *et al*., **1986**)	**Rods; Pink**	**NA**	**NA**	**2.5**	**NA**	**Acid mine drainage**
Acidiphilium cryptum (**Harrison, 1981**)	**Rods; White to pink**	**NA**	**27-35**	**3-4**	**NA**	**Coal mine refuse, copper mine effluent**
Acidiphilium multivorum (**Wakao** *et al*., **1994**)	**Rods; White to pink**	**NA**	**27-35**	**3-4**	**NA**	**Pyritic acid mine drainage**
Acidiphilium organovorum (**Lobos** *et al*., **1986**)	**Bacilli or cocco-bacilli; White**	**NA**	**30-42**	**3**	**NA**	**Acidic mineral waters**
Acidiphilium rubrum (**Wichlacz** *et al*., **1986**)	**Rods; Reddish violet**	**792, 864**	**27-35**	**3-4**	**NA**	**Acid mine drainage**
Acidisphaera rubrifaciens (**Hiraishi** *et al*., **2000a**)	**Cocci or cocco-bacilli; Pink to red**	**801, 873-874**	**30-35**	**4.5-5**	**NA**	**Acidic hot springs and pyritic mine drainage**
Blastomonas natatoria (**Hiraishi** *et al*., **2000b**)	Ovoid to rod; Yellow, orange or brown	NA	30-35	7-7.5	NA	Freshwater environments
Citromicrobium bathyomarinum (**Yurkov** *et al*., **1999**)	**Pleomorphic; Yellow**	**800, 867**	**20-42**	**6-8**	**1-5**	**Deep ocean hydrothermal vent plume**
Congregibacter litoralis (Fuchs *et al*., 2007)	Pleomorphic; Pink	NA	NA	NA	NA	Ocean surface
Craurococcus roseus (Saitoh *et al*., 1998)	Cocci; Pink	800, 872	28-32	7.5	0-0.1	Soil
Dinoroseobacter shibae (Biebl *et al*., 2005)	Cocci to ovoid; Beige to light red	804, 868	33	6.5-8.8	1-$\geq$7 [2]	Marine dinoflagellate culture
Erythrobacter litoralis (**Yurkov** *et al*., **1994b**)	**Rods; Orange to brown**	**800, 868**	**25-30**	**7-8.5**	**0.5-1.5**	**Marine microbial mat with fluctuating salinity**
Erythrobacter longus (Shiba and Simidu, 1982)	Rods; Orange	807, 866	30	7	1.7-3.5	Ocean surface

Table 1 continued.

Erythromicrobium ezovicum (Yurkov *et al.*, 1991)	**Rods; Reddish orange**	**798, 836, 868**	**25-30**	**7-8**	**NA**	**Alkaline spring**
Erythromicrobium hydrolyticum (Yurkov *et al.*, 1991)	**Rods; Reddish orange**	**798, 840, 868**	**25-30**	**7-8**	**NA**	**Alkaline spring**
Erythromicrobium ramosum (Yurkov *et al.*, 1994b)	**Rods; Reddish orange**	**798, 832, 870**	**25-30**	**7-8.5**	**NA**	**Alkaline spring**
Erythromonas ursincola (Yurkov *et al.*, 1997)	**Ovoid; Orange to orangish brown**	**801, 853, 867**	**25-30**	**7-8**	**NA**	**Thermal spring**
Geminicoccus roseus (Foesel *et al.*, 2007)	Diplococcic; Whitish grey to pink	NA	30-35	8	0.25-1	Marine aquaculture biofilter
Hoeflea phototrophica (Biebl *et al.*, 2006)	Short rod with capsule; Colorless to pink	NA	31	6-9	0.5-$\geq$7 [2]	Marine dinoflagellate culture
Labrenzia alexandrii (Biebl *et al.*, 2007)	Rods with uneven ends; Faint pink	801,865	26	7-8.5	1-7	Marine dinoflagellate culture
Marivita cryptomonadis (Hwang *et al.*, 2009)	Rods; Creamy	NA	30	7-9	3-5	Marine phytoplankton culture
Marivita litorea (Hwang *et al.*, 2009)	Rods; Creamy	NA	30	7-8	3-5	Coastal seawater
Paracraurococcus ruber (Saitoh *et al.*, 1998)	Cocci; Red	802, 856	30-34	6.6-6.8	0-0.1	Soil
Porphyrobacter cryptus (Rainey *et al.*, 2003)	**Rods; Reddish orange**	**800, 870**	**50**	**7.5-8.5**	**NA**	**Thermal spring**
Porphyrobacter dokdonensis (Yoon *et al.*, 2006)	Pleomorphic: cocci, ovoid or rod; Reddish orange	800, 835, 862	35-37	7-8	2	Sea water
Porphyrobacter donghaensis (Yoon *et al.*, 2004)	Pleomorphic: cocci, ovoid or rod; Reddish orange	808, 867	30-37	7-8	2	Sea water
Porphyrobacter meromictius (Rathgeber *et al.*, 2007)	**Short rod or pleomorphic; Reddish brown**	**806-808, 866-867**	**10-37 [2]**	**5.5-10 [2]**	**0-2**	**Meromictic lake**
Porphyrobacter neustonensis (Fuerst *et al.*, 1993)	Pleomorphic rods and cocci; Orange or red	799-806, 868-871	20-30	8		Freshwater pond surface

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Table 1 continued.

Porphyrobacter sanguineus (Hiraishi *et al.*, 2002)	Rods and pleomorphic; Orange or red	799, 814, 861	30	7-7.5	1	Brackish and marine
Porphyrobacter tepidarius (Hanada *et al.*, 1997)	**Ovoid to short rods; Orange**	**800, 870**	**40-48**	**6.5-8.5**	**0**	**Thermal spring**
Roseateles depolymerans (Suyama *et al.*, 1999)	Rods; Pink	800, 870	35	6.5	NA	River
Roseibaca ekhonensis (Labrenz *et al.*, 2009)	**Rods; Red**	**865-866**	**16**	**7-9.5**	**2.5**	**Hypersaline heliothermal meromictic lake**
Roseibacterium elongatum (Suzuki *et al.*, 2006)	Rods, variable length; Pink	800, 879	27-30	7.5-8	0.5-7.5 [2]	Sand, shark bay
Roseibium denhamense (Suzuki *et al.*, 2000)	Rods; Pink	803-805, 863-864	27-30	7.5-8.5	0.5-10 [2]	Marine intertidal flats
Roseibium hamelinense (Suzuki *et al.*, 2000)	Rods; Pink	803-805, 872-873	27-30	7.5-8	0-10 [2]	Marine intertidal flats
Roseicyclus mahoneyensis (Rathgeber *et al.*, 2005)	**Pleomorphic: ovoid, long rod, vibrio, cyclical rod; Pinkish purple to purple**	**805-806, 870-871**	**4-37 [2]**	**6->11 [2]**	**0.5-10 [2]**	**Meromictic lake**
Roseinatronobacter thiooxidans (Sorokin *et al.*, 2000)	**Lemon-shaped rods; Pink to reddish orange**	**803, 870**	**NA**	**10**	**3.8-5.7**	**Alkaline, hypersaline lake**
Roseinatronobacter monicus (Boldareva *et al.*, 2007)	**Ovoid; Pink**	**804, 870**	**25-30**	**8.5-9.5**	**4**	**Hypersaline lake**
Roseisalinus antarcticus (Labrenz *et al.*, 2005)	**Rods; Red**	**800-801, 870**	**16-26**	**7-7.8**	**5-9**	**Meromictic lake**
Roseivivax halodurans (Suzuki *et al.*, 1999a)	**Rods; Pink**	**803, 873**	**27-30**	**7.5-8**	**0.5-20 [2]**	**Saline lake**
Roseivivax halotolerans (Suzuki *et al.*, 1999a)	**Rods; Pink**	**805, 871**	**27-30**	**7.5-8**	**0-20 [2]**	**Saline lake**
Roseobacter denitrificans (Shiba, 1991)	Rods; Pink	805-807, 868-873	20-30	7.5-8	NA	Ocean surface
Roseobacter litoralis (Shiba, 1991)	Rods; Pink	805-807, 868-873	20-30	7.5-8	NA	Ocean surface

Table 1 continued.

Roseococcus suduntuyensis **(Boldareva *et al*., 2009)**	**Cocci or short rods; Pink**	**865**	**20-35**	**8.5-9.5**	**2-5**	**Soda lake**
Roseococcus thiosulfatophilus **(Yurkov *et al*., 1994b)**	**Cocci; Pinkish red**	**800, 855**	**25-30**	**7-8**	**NA**	**Thermal alkaline spring**
Roseovarius tolerans **(Labrenz *et al*., 1999)**	**Rods; Red, pink, beige, whitish beige**	**799-802, 877-879**	**9-34**	**6.9-9**	**1-13**	**Hypersaline heliothermal meromictic lake**
Roseovarius mucosus (Biebl *et al*., 2005a)	Ovoid; Pink	NA	31	6.5-8.8	1-7	Marine dinoflagellate culture
Rubrimonas cliftonensis **(Suzuki *et al*., 1999b)**	**Short rods; Pink**	**806, 871**	**27-30**	**7.5-8**	**0.5-7.5 [2]**	**Saline lake**
Rubritepida flocculens **(Alarico *et al*., 2002)**	**Very short rods; Red**	**800, 855**	**50**	**7-8**	**NA**	**Thermal spring**
Sandaracinobacter sibiricus **(Yurkov *et al*., 1997)**	**Long thin rods; Yellowish orange**	**800, 867**	**25-30**	**7.5-8.5**	**0**	**Thermal spring**
Sandarakinorhabdus limnophila (Gich and Overmann, 2006)	Rods; Reddish orange	800, 837, 865	NA	NA	NA	Freshwater lake
Sphingomonas kaistensis (Kim *et al*., 2007)	Rods; Reddish orange	NA	25-30	NA	NA	Soil
Staleya guttiformis **(Labrenz *et al*., 2000)**	**Rods; Beige to pink**	**800-802, 861-862**	**12-20**	**7-8.5**	**1-4**	**Hypersaline heliothermal meromictic lake**
Stappia marina (Kim *et al*., 2006)	Rods; color NA	NA	NA	NA	3	Tidal flat
Stappia stellulata (Rüger and Höfle, 1992; Biebl *et al*. 2007)	Rods; White to brown	NA	20-30	NA	NA	Sea water and marine sediment
Thalassobacter stenotrophicus (Macián *et al*., 2005)	Ovoid to irregular rods; Salmon-pink	NA	22-26	NA	0.85-7 [2]	Sea water

Boldface indicates organisms from extreme environments.

[1] NA = not available.

[2] full range of tolerance, not optimum.

The AAP are closely allied with, and probably descended from purple nonsulfur bacteria, as demonstrated by small subunit (5S and 16S) rRNA analyses, DNA-DNA hybridization, G+C content and 16S-23S internal transcribed spacer sequence analysis (Yurkov and Csotonyi, 2009). However, AAP are not a homogeneous group, but are phylogenetically interspersed with phototrophic and strictly heterotrophic *Proteobacteria* (Rathgeber *et al*., 2004; Yurkov and Csotonyi, 2009). Genetic interspersion occurs at two levels, via photosynthetic apparatus genes and 16S rRNA genes, both providing useful insight into their evolutionary topology.

Although 16S rRNA gene sequence similarity indicates that AAP from each proteobacterial class (*Alpha-*, *Beta-* and *Gammaproteobacteria*) descended from purple phototrophs in their respective classes, photosynthesis genes (*puf* and *puc*, encoding the proteins of the RC and LH complexes) are shuffled between classes, probably by lateral gene transfer (Yurkov and Csotonyi, 2009). For example, betaproteobacterial *Roseateles depolymerans* is closely related to purple nonsulfur *Rubrivivax gelatinosus*, but its entire 37-kb photosynthetic gene cluster is alphaproteobacterial (Igarashi *et al*., 2001). Even more strikingly, strain JL354 (a close relative of alphaproteobacterial *Citromicrobium bathyomarinum*) possesses both a complete alphaproteobacterial photosynthetic operon and a partial gammaproteobacterial version (Jiao *et al*., 2010). However, marked differences of AAP LH complexes from those of purple bacteria implies that lateral gene transfer only occurred within the AAP after evolutionary divergence from purple bacteria (Yurkov and Csotonyi, 2009).

According to 16S rRNA gene sequences, several AAP clades subtend or are closely related to organisms lacking a photosynthetic apparatus (e.g. *Labrenzia alexandrii*, which expresses BChl and possesses *puf* genes, and *Labrenzia aggregata*, which has none) (Biebl *et al*., 2007). Close affinity with non-phototrophs has led several authors to assign novel non-

phototrophic species to AAP genera, especially in the cases of *Erythrobacter*, *Roseovarius* and *Roseobacter* (Yurkov and Csotonyi, 2009). Some AAP genera (e.g. *Roseivivax*) are even emended to make them ambiguous regarding BChl production to accommodate non-phototrophic species (Park *et al*., 2010). This practice introduces chaos into taxonomy, and a solution is needed. Nevertheless, it reflects an important evolutionary relationship between AAP and their relatives. Many non-phototrophs appear to have evolved from AAP ancestors by loss of phototrophy. Perhaps all strictly heterotrophic *Proteobacteria* descended from anoxygenic phototrophs (Woese, 1987).

Prior to a deeper discussion of the characteristics across which AAP are divided from their purple bacterial progenitors, the perplexing existence of gammaproteobacterial AAP deserves treatment. The greater aerotolerance and more versatile photoheterotrophic nutritional mode of purple nonsulfur bacteria than purple sulfur bacteria leads to the hypothesis that the roots of AAP lie more immediately within the former group. Therefore, the (thus far reported) absence of gammaproteobacterial purple nonsulfur bacteria probably explains the general restriction of AAP to *Alpha-* and *Betaproteobacteria*. It appears that gammaproteobacterial AAP such as *C. litoralis* are polyphyletic with respect to other AAP, a conclusion supported by their uniquely organized photosynthetic operon (see Beja *et al*., 2002 and Fuchs *et al*., 2007). However, burying their origins in even deeper mystery is the fact that *C. litoralis* is not even closely related to any known gammaproteobacterial purple sulfur bacteria (e.g. *Ectothiorhodospira* and *Chromatium*), residing rather within the generally non-phototrophic NOR5/OM60 clade between *Alteromonadaceae* and *Pseudoalteromonadaceae* (Spring *et al*., 2009). This begs the question of whether gammaproteobacterial AAP represent the descendents of an unknown lineage of phototrophs with thus far no cultured extant anaerobic members, or

whether they are the result of an unusually distant transfer of the photosynthesis gene cluster

from phototrophs of one bacterial class to non-phototrophs of another. An uninterrupted

nucleotide signature of the photosynthetic superoperon of *C. litoralis* and the absence of

transposons in its vicinity argue against a recent lateral gene transfer event (Fuchs *et al.*, 2007).

Either alternative origin hypothesis has significant implications for the evolution and

phylogenetic distribution of photosynthesis and AAP. Discovery and investigation of additional

representatives of this group (Chapter 3) will not only contribute greatly to fleshing out the

phenotypic diversity of the clade but may also provide invaluable insight into their obscure

evolutionary descent.

1.3.2. Traits that ally and distinguish AAP from phototrophic purple bacteria

The AAP exhibit several "ordinary" anoxygenic phototrophic traits, typical of purple

phototrophs. They share a similar organization of the photosynthetic operon and apparatus,

consisting of a type II (quinone-type) RC, surrounded by LH complexes whose added area and

integrated carotenoids increase the number and wavelength range of photons captured (Yurkov

and Csotonyi, 2009). The RC in both AAP and anaerobic phototrophs has L, M and H protein

subunits, four BChl, a pair of bacteriopheophytins, two ubiquinones (Q_A and Q_B), a nonheme

high-spin Fe^{2+}, and a carotenoid (Yurkov and Beatty, 1998a). The molar ratios of RC pigments

and electron carriers are similar to those in purple nonsulfur bacteria (Takamiya *et al.*, 1987).

Like purple phototrophs, AAP also possess a transmembrane cytochrome bc_1 complex

downstream from the RC and sometimes a purple bacterial type tetrahemic RC-bound

cytochrome *c* (Yurkov and Beatty, 1998a). Similarity in RC gene sequences of AAP and

anaerobic phototrophs (Nagashima *et al.*, 1997) suggests that only a few modifications were

required to allow for aerobic phototrophy. Absorption spectra, reduction-oxidation difference spectra, and the photochemistry of the RC also resemble the purple nonsulfur bacteria (Yurkov *et al.* 1998). Furthermore, quantum yield of energy transfer from carotenoids to LHII of the AAP *E. ramosum* matches values for *Rhodobacter sphaeroides* (Yurkov *et al.*, 1994a).

Here ends the resemblance of AAP to purple phototrophs. Their namesake feature, the incapacity for anaerobic photosynthesis, results from up to four deviations from purple phototrophs. Probably most importantly, an anomalously high redox midpoint potential of the primary electron acceptor Q_A in the photochemical electron transport chain becomes over-reduced anaerobically, arresting electron flow (Rathgeber *et al.*, 2004). Second, soluble cytochrome *c* fails to transfer electrons anoxically from the cytochrome bc_1 complex to the RC-bound cytochrome *c* (Schwarze *et al.*, 2000). Third, AAP lack a Calvin cycle (Yurkov and Beatty, 1998a), and finally, they have no quinol oxidase pathway (Rathgeber *et al.*, 2004).

In contrast to purple bacteria, BChl biosynthesis in AAP is inhibited by light, whereas oxygen is required for growth and pigment production. The O_2 regulation of BChl synthesis may be due to an O_2-requiring Mg-protoporphyrin monomethyl ester cyclase instead of the H_2O-requiring version that functions anaerobically in purple bacteria (Ouchane *et al.*, 2004). This enzyme catalyzes the conversion of key intermediates in the BChl synthesis pathway (Mg-protoporphyrin monomethyl ester to Mg-divinyl protochlorophyllide *a*). However, presence of both versions of the gene in the AAP *Roseobacter denitrificans* (Swingley *et al.*, 2007), and the low optimal O_2 concentration for BChl synthesis or growth in *R. depolymerans* (Suyama *et al.*, 2002), *Roseinatronobacter thiooxidans* (Sorokin *et al.*, 2000), *C. litoralis* (Fuchs *et al.*, 2007) and *Roseicyclus mahoneyensis* (Rathgeber *et al.*, 2005) indicates that our understanding is still incomplete.

Although moderate illumination maximally stimulates photosynthetic pigment synthesis in purple nonsulfur bacteria, even low light intensity paradoxically inhibits BChl production in AAP (Yurkov and Beatty, 1998a), destabilizing the pigment before it can be incorporated into protein complexes (Iba and Takamiya, 1989). Hence, AAP are adapted to an alternating light/dark cycle, diurnally utilizing BChl that is synthesized during the previous night (Biebl and Wagner-Döbler, 2006; Yurkov and Van Gemerden, 1993b). Even in the dark, AAP typically produce at least 5 times less BChl than anaerobic phototrophic bacteria, from 0.0023 nmol/mg dry weight to 4.0 nmol/mg of protein depending on the species (Hiraishi *et al.*; 1998, Yurkov, 2006). Low BChl content and inhibition by light may protectively minimize the incidental production of toxic triplet BChl in the presence of light and O_2 (Beatty, 2002), a bigger problem for AAP than for their anaerobic counterparts.

Although the RC of AAP resembles that of purple bacteria, their LH complexes differ significantly, especially in the protein environment of BChl and in accessory LHII complexes. Compared to the 800 and 850 nm absorbance peaks of the typical purple bacterial LHII (Imhoff, 2001a), those of *Erythromicrobium*, *Sandarakinorhabdus* and *Porphyrobacter dokdonensis* absorb at 798 and 832 nm (Yurkov and Beatty, 1998a; Gich and Overmann, 2006; Yoon *et al.*, 2006), while that of *Porphyrobacter donghiensis* absorbs at 800 and 814 nm (Yoon *et al.*, 2004). In contrast, the unusual LHII of *R. denitrificans*, *R. cliftonensis* and *R. mahoneyensis* absorbs monomodally at about 806 nm (Suzuki *et al.*, 1999b; Rathgeber *et al.*, 2005). The protein environment of BChl in *R. thiosulfatophilus* causes its LHI peak to be blue-shifted to 856 nm, compared to 870 nm for most purple phototrophs (Yurkov *et al.*, 1994a). By contrast, similar blue-shifting in acidophilic AAP species of *Acidiphillium* and *Acidisphaera* is caused by the

unique replacement of Mg with Zn in the porphyrin ring of BChl *a* (Hiraishi and Shimada, 2001).

AAP also differ from purple phototrophs in the composition and distribution of their carotenoids. The bright colors of AAP are due to the great abundance of these pigments in AAP (Yurkov and Csotonyi, 2009). Most AAP contain a different suite of carotenoids than purple bacteria, with carotenes often giving them more orange hues (Yurkov, 2006). AAP are also unique among phototrophs in possessing highly polar carotenoid sulfates and C_{30} carotenoid glucosides (Yurkov *et al.*, 1993). Enrichment in carotenoids leads to BChl/carotenoid absorption ratios of only 1:8 to 1:10 (Yurkov, 2006). Interestingly, the majority of carotenoids in AAP are located in the peripheral membrane fractions and cell wall, where they are decoupled from photosynthetic activity (Noguchi *et al.*, 1992, Yurkov *et al.*, 1993). These unbound carotenoids may shield cells from intense light or scavenge reactive singlet oxygen or triplet BChl that can damage cell components (Noguchi *et al.*, 1992), analogous to but more pronounced than in anaerobic phototrophs (Fraser *et al.*, 2001).

1.3.3. Global distribution and ecological niche of AAP

The first AAP were discovered in the ocean (Shiba *et al.*, 1979). Many α-3 proteobacterial *Roseobacter* clade members, like their namesake genus, inhabit nutrient-poor marine environments. Beatty (2002) has proposed that AAP are particularly well adapted to life in oligotrophic habitats. Their phototrophic metabolism, which competes for a common electron transport chain with respiration (Yurkov *et al.*, 1993), appears to be most active when carbon sources are limiting (Suyama *et al.*, 2002; Biebl and Wagner-Döbler, 2006). This gives AAP a competitive edge exactly when it is most useful, i.e. when strict heterotrophs are most starved

(Beatty, 2002). AAP have also been recovered from numerous other habitats. Of the mesic species, α-1 proteobacterial *Craurococcus* and *Paracraurococcus* were isolated from Japanese soils (Saitoh *et al*., 1998), but AAP have recently been found in xeric Canadian soil crusts as well (Csotonyi *et al*., 2010). Several α-4 proteobacterial genera (e.g. *Erythrobacter*, *Erythromicrobium*, *Porphyrobacter*) are native to eutrophic freshwater and marine microbial mats (Rathgeber *et al*., 2004; Yurkov and Beatty, 1998a; Yurkov and Csotonyi, 2003; 2009) (Table 1). A fosmid library from the Delaware River suggested that betaproteobacterial members might be particularly abundant in river systems (Waidner and Kirchman, 2005).

Much of the recent work on global AAP distribution has focused on the most extensive oligotrophic habitat, the ocean, where they contribute substantially to microbial abundance, biomass and the marine carbon budget (Fenchel *et al*., 1998, Kolber *et al*., 2000, Kolber *et al*., 2001; Goericke, 2002). AAP constitute anywhere from 0.01% to 34% of total marine bacterioplankton abundance, depending upon a host of factors, including season of sampling, water temperature and primary productivity (Cottrell *et al*., 2006; Koblížek *et al*., 2003; Lami *et al*., 2007; Schwalbach and Fuhrman, 2005; Sieracki *et al*., 2006; Waidner and Kirchman, 2007; Yurkov and Csotonyi, 2009). Most likely, a sizable proportion of this variation is also explained by technical challenges in the estimation of AAP abundance. These problems include the confusion of AAP with anaerobic phototrophs (if BChl expression is used to assay AAP abundance) and with non-phototrophs (when abundance is determined by phylogenetic analyses alone) (Yurkov and Csotonyi, 2009).

Although AAP cannot fix inorganic carbon above anapleurotic (TCA cycle intermediate) rates, they can still increase the productivity of microbial communities by utilizing light for energy. In fact, photophosphorylation increases electron fluxes three-fold over respiration in

marine strain COL2P, related to *Roseobacter* (Koblížek *et al.*, 2010). Similarly,

Erythromicrobium hydrolyticum acquires about 10% of its cellular energy needs from

photophosphorilation (Yurkov and van Gemerden, 1993b). As a result, AAP can divert some of

their acquired carbon from an anabolic to a catabolic role (Yurkov and van Gemerden, 1993b;

Sieracki *et al.*, 2006). AAP may also play significant roles in sulfur metabolism (Yurkov *et al.*

1994c; González *et al.*, 1999; Wagner-Döbler and Biebl, 2006), and lignin or aromatic carbon

compound degradation (Buchan *et al.*, 2000; 2001). Some species have been implicated as

symbionts of cuttlefish (Grigioni *et al.*, 2000), oysters (Boettcher *et al.*, 2000), dinoflagellates

(Hold *et al.*, 2001), diatoms (Schäfer *et al.*, 2002) and red algae (Ashen and Goff, 2000).

Although researchers have only scratched the surface of the ecological niches of AAP,

they have observed the interesting trend that AAP show considerable competence in the face of

physically stressful conditions. First, AAP upregulate photosynthetic pigment production when

starved (Suyama *et al.*, 2002) or cultured under suboptimal temperature (Alarico *et al.*, 2002) and

salinity (Rathgeber *et al.*, 2004; Macián *et al.*, 2005; Biebl *et al.*, 2006). Because photosynthesis

can amend the energetic requirements of AAP, its overexpression under conditions near their

survival limits can increase competitive potential relative to the rest of the community in

marginal environments. Second, Jiao *et al.* (2004), used flow cytometry with a 3,3'-

dihexyloxacarbocyanine iodide chemical probe to demonstate that the membrane potential of a

Roseobacter clade strain, DG26-Y-1, was less sensitive to fluctuations in temperature and pH

than that of a strictly heterotrophic proteobacterium, *E. coli*. Because membrane potential is an

indicator of the proton motive force, it reflects membrane integrity, energy status and cell

viability (Jiao *et al.*, 2004), implying that even AAP from mesic environments such as the ocean

are predisposed to high stress tolerance. The above results may help explain why AAP are especially prominent components of numerous extreme environments.

1.4. Aerobic anoxygenic phototrophs in hydrothermal and hypersaline environments

Out of 57 currently described species of AAP, 28 were isolated from extreme environments or else exhibit extremophily or extremotolerance. Although sampling bias may be responsible for some of this large fraction because of the growing popularity of extreme environments, the proportion is nevertheless high. AAP have been studied in five major types of stressful habitats: (1) terrestrial and deep ocean hydrothermal systems (Yurkov and Beatty, 1998a; 1998b), (2) hypersaline biotopes that include springs (Csotonyi *et al*., 2008), supralittoral microbial mats (Yurkov and van Gemerden, 1993a) and meromictic lakes (Yurkova *et al*, 2002), (3) alkaline soda lakes (Sorokin *et al*., 2000), (4) acidic mine drainage waters (Hiraishi and Shimada 2001) and most recently, (5) biological soil crusts on xeric soils (Csotonyi *et al*., 2010). This review addresses the first two types of environments, reflecting the focus of the project.

There is some phylogenetic stratification of AAP among extreme environments. All known acidophiles are α-1-proteobacterial members of *Acidiphilium* and *Acidisphaera* (Hiraishi and Shimada, 2001). Marine and hypersaline environments are especially rich in α-3-proteobacterial representatives in the '*Roseobacter* clade'. Hydrothermal systems possess both α-1-proteobacterial species (*Roseococcus* and *Rubritepida*) and the tightly-knit α-4-proteobacterial cluster (e.g. *Citromicrobium*, *Erythrobacter*, *Erythromicrobium*, *Erythromonas*, *Porphyrobacter*) (Yurkov and Csotonyi, 2009).

1.4.1. Hydrothermal systems: terrestrial and deep ocean

Hydrothermal vents are characterized by efflux of water from subterranean geothermally heated aquifers (Kelley *et al*., 2002). Depending on geological properties, the temperature of effluent solutions can range from near ambient to boiling (about 100°C at earth's surface to nearly 400°C at some deep ocean locations) (Delaney *et al*., 1992). Many hydrothermal vents release prodigious reduced compounds such as H_2S, supporting a rich microbial community.

1.4.1.1. Terrestrial hydrothermal systems

In terrestrial systems that overly anaerobic anoxygenic phototrophic mats, and where atmospheric mixing has entrained O_2 into the water, ten species of AAP in seven genera have been isolated (Table 1). These organisms rely on reduced carbon fixed by the resident anaerobic anoxygenic phototrophs, chemolithoautotrophs, and oxygenic phototrophs such as cyanobacteria and algae. The first described non-marine AAP, *Erythrobacter sibiricus* (later renamed *Sandaracinobacter sibiricum*), was recovered from the Bol'shaya River thermal springs, from an ambient temperature of 54°C (Yurkov and Gorlenko, 1990). Other terrestrial species hail from thermal springs in Japan (*Porphyrobacter tepidarius*) (Hanada *et al*., 1997), Portugal (*Porphyrobacter cryptus*) (Rainey *et al*., 2003), Hungary (*Rubritepida flocculens*) (Alarico *et al*., 2002) and Russia (*Erythromonas ursincola*, *Sandaracinobacter sibiricus* and three species of *Erythromicrobium*: *E. ezovicum*, *E. hydrolyticum* and *E. ramosum*) (Yurkov and Gorlenko, 1990; Yurkov *et al.*, 1991; 1994b). The species *P. cryptus*, *P. tepidarius* and *R. flocculens* show significant extremophily or extremotolerance, in accordance with their high native habitat temperatures (42.7 to 53°C). The maximum tolerable temperature reported for AAP is 55°C (*P. cryptus*; Rainey *et al*., 2003), which is considerably lower than the 73°C that some oxygenic

phototrophs can survive (Miller and Castenholtz, 2000). Low solubility of oxygen in hot water is probably partly responsible for this phenomenon, since aeration stimulates the growth of AAP (Yurkov and Beatty, 1998a). Temperature affects not only the survival of hydrothermal vent AAP, but also pigment production in some species. Interestingly, *R. flocculens* synthesizes no BChl at its optimum temperature of 50°C, even though it does so at 30°C (Alarico *et al*., 2002). Marine *Erythrobacter* relative NAP1 grew optimally at 32.5^{o}C, but BChl production maximized at 22.5^{o}C and was absent at temperatures above 30°C (Koblížek *et al*., 2003). The increase in pigment production under suboptimal growth conditions (Rathgeber et al., 2004), allows AAP to perform better in unusual conditions than they would with strict chemotrophy.

1.4.1.2. Deep ocean hydrothermal vents

The most unusual environment from which any phototrophic organism has been isolated is the sunless realm of deep ocean hydrothermal vent plume waters. The sulfide-rich water in the vicinity of black smoker chimneys supports a diverse chemolithotrophic microbial community, forming the basis of one of the few ecosystems that derive energy mainly from geothermal processes instead of solar radiation. Amazingly, however, radiation is not entirely absent at deep ocean black smoker chimneys. Within 5 cm of the vent orifice, up to 1.5×10^{11} photons/cm^{2}/s may be generated in the 950-1050 nm range (Van Dover et al., 1994; 1996).

The curious overlap between black smoker emission spectra and the action spectrum of BChl prompted the idea that anoxygenic photosynthesis may have arisen at hydrothermal vents (Nisbet *et al*., 2002). In fact, vent radiation output is comparable to minimal photosynthetic requirements of green sulfur bacteria (Overmann *et al*., 1992). Therefore, discovery of the deep ocean AAP *Citromicrobium bathyomarinum* (Yurkov and Beatty, 1998b) and the green sulfur

bacterium, *Chlorobium* strain GSB1 (Beatty *et al*., 2005), was foreseeable, if remarkable. GSB1 appears to be obligately photosynthetic (Beatty et al., 2005), suggesting that it must live near the vent orifice. By contrast, *Citromicrobium* was isolated from 2000 m deep vent plume waters of the Endeavor segment of the Juan de Fuca Ridge (Yurkov and Beatty, 1998b). Its pelagic niche is consistent with the primarily heterotrophic metabolism of AAP. Enumeration of AAP from a vertical transect above the Endeavor vent field has confirmed that this species is confined to the deep ocean (Rathgeber *et al*., 2008). Seven strains formed a tight phylogenetic cluster with *C. bathyomarinum*, while an eighth isolate, from near-surface waters, was more closely affiliated with *Erythrobacter*, *Erythromicrobium* and *Porphyrobacter* species. Amazingly, despite its low pigment content (at most 0.6 nmol BChl per mg protein; Yurkov *et al*., 1999), *C. bathyomarinum* produces a photochemically functional photosynthetic apparatus (Rathgeber *et al*., 2008). This would imply that some populations of *C. bathyomarinum* must inhabit a niche sufficiently proximal to high temperature venting to exploit geothermal illumination as a source of energy. The abundant carotenoids of AAP have been suggested to confer protection from oxidative damage (Yurkov and Csotonyi, 2009), possibly ameliorating the effects of toxic heavy metal oxides commonly found in deep ocean hydrothermal environments (also see Chapter 7). *C. bathyomarinum* is well suited to tolerating other extremes that it may experience near vents. This species is one of the most thermotolerant and halotolerant of AAP, growing at temperatures between 4 and 45$^{\text{o}}$C, and at NaCl concentrations between 0 and 10% (Yurkov *et al*., 1999). *C. bathyomarinum* can also grow at pH between 5 and 10, a range matched among AAP only by *A. acidophilum* (Hiraishi *et al*., 1998). These adaptations would promote survival under the changing physico-chemical conditions that prevail as a hydrothermal vent evolves from origin to extinction (Kadko and Butterfield, 1998; Van Dover *et al*., 1994).

1.4.2. Hypersaline environments

Hypersaline environments, where salinity exceeds marine values of about 3% (Rodriguez-Valera, 1992), have made the greatest recent contribution to novel AAP taxonomy: 13 species in 11 genera (Table 1), and many isolates await formal classification. The salinity of their habitats reaches up to at least 17% (Ekho Lake, Antarctica; Labrenz and Hirsch, 2001). Halotolerance and optimal salinity of AAP from hypersaline systems reflect this range of salt concentration. For example, *Staleya guttiformis*, from Ekho Lake, can grow between 0 and 20% NaCl (Labrenz *et al*., 2000), and strain EG11 from the EGC springs displays the greatest known salinity tolerance for any AAP, from 0 to 26% NaCl (Csotonyi *et al*., 2008). Hypersaline ecosystems fall into three broad categories: (1) supralittoral marine pools and flats with temporally fluctuating salinity (Shiba *et al*., 1979; Yurkov and van Gemerden, 1993a), (2) saline and meromictic lakes in which salinity is spatially stratified along a vertical gradient (Labrenz and Hirsch, 2001; Yurkova *et al*., 2002) and (3) hypersaline springs, with unidirectional flow (Csotonyi *et al*., 2008).

1.4.2.1. Supralittoral marine environment.

Halotolerant AAP were first isolated by Shiba *et al*. (1979) from intertidal zones on the east and west coasts of Australia. Tidal pools experience fluctuating salinity during lunar and diurnal cycles. Between periods of inundation, desiccation raised salinity up to 6.6% in the Australian pools (Shiba *et al*., 1979), and up to 10% in a supralittoral microbial mat on the coast of the Dutch island of Texel (Yurkov and van Gemerden, 1993a). AAP were considerably more frequent in Australian hypersaline communities (10 to 49% of the microbial community) than in

Japanese marine communities (typically 1%) (Shiba *et al.*, 1996), suggesting higher competitive ability of AAP than most other microorganisms under stressful conditions. Meanwhile, AAP constituted 6.7% of the anoxygenic phototrophic community in the Texel mats, equal in number to purple nonsulfur bacteria (Yurkov and van Gemerden 1993a). Despite lower frequency, the AAP abundance at Texel was about ten-fold greater than at the Australian sites (Shiba *et al.*, 1979), consistent with greater organic matter content of the well-developed Texel microbial mat (Yurkov and van Gemerden, 1993a). The Texel community yielded halotolerant *Erythrobacter litoralis*, capable of growth between 0.5 and 9.6% salinity (Yurkov *et al.*, 1994b).

1.4.2.2. Meromictic and saline lakes.

Unlike tidal pools, meromictic lakes display primarily spatial rather than temporal variation in salinity. Salt concentration can increase drastically between the surface (e.g. 0.9%) and a few meters depth (e.g. 6.5%) (Hall and Northcote, 1986). High salt content can create persistent vertical stratification of water density, the lower layers resisting turnover and isolating the chemical environment of the surface from that of the benthos (Northcote and Halsey, 1969). Anoxic conditions that develop at depth promote growth of sulfate reducing communities, which drive blooms of purple sulfur bacteria (Overmann *et al.*, 1991; 1994; 1996). Hypersaline meromictic lakes are often heliothermal: low specific heat capacity and solar heating can generate temperatures of $60^{o}C$ (Javor, 1989). As a result, meromictic lake microorganisms typically display multiple tolerances to pH, temperature and salinity (Labrenz and Hirsch, 2001).

Exemplifying the great range in environmental factors of meromictic lakes, *R. tolerans* from Antarctic meromictic Ekho Lake tolerates osmolarity of 1% to over 15%, pH of 5.3 to over 9.0, and temperatures from $<3^{o}C$ to $43.5^{o}C$ (Labrenz *et al.*, 1999). Other α-3-proteobacterial

AAP from Ekho Lake, *S. guttiformis* (Labrenz *et al.*, 2000) and *Roseibaca ekhonensis* (Labrenz *et al.*, 2009), exhibit similar physiological flexibility.

Unlike thalassohaline Ekho Lake, which is thought to be of marine origin (Labrenz *et al.*, 1999), Mahoney Lake in British Columbia, Canada derives its salinity from the surrounding lavas, making it an athalassohaline (not of marine origin), sodium sulfate dominated meromictic lake (Yurkova *et al.*, 2002), with a vertical salinity gradient ranging from 4 to 40‰ (Hall and Northcote, 1986). Not surprisingly, Mahoney Lake AAP displayed broad tolerance to NaCl (0 to 10%), Na_2SO_4 (0 to 14%) and pH (5.5 to 11) (Yurkova *et al.*, 2002). AAP from the surface, 3 and 5 m depths within the lake constituted 12%, 4% and 6% of all aerobically cultured bacteria, respectively (Yurkova *et al.*, 2002). These are similar to the proportions reported by Shiba *et al.* (1979) for saline and hypersaline environments on Australian coasts.

Buttressing the repeated observation that extreme environments host high AAP diversity, 31 Mahoney Lake isolates displayed a wide range of morphological, phylogenetic, photosynthetic and physiological characteristics. Based on a partial 16S rRNA gene sequence analysis, they clustered into 17 groups, with nearest relatives scattered among a variety of AAP and heterotrophic α-proteobacterial taxa (Yurkova *et al.*, 2002). Although more species await description, Mahoney Lake has already yielded the α-3-proteobacterial *Roseicyclus mahoneyensis* (Rathgeber *et al.*, 2005) and the α-4-proteobacterial *Porphyrobacter meromictius* (Rathgeber *et al.*, 2007). The former species stands apart because it possesses a highly curved vibrioid morphology unique among AAP and an unusual *Roseobacter*-like monomodal LHII (λ_{max} = 806 nm). *Roseicyclus* can only be cultured on complex growth medium, suggesting an adaptation to utilizing specific nutrients, such as humic substances (Rathgeber *et al.*, 2005).

Although a quantitative estimate of AAP diversity is not yet available for most extreme environments, Jiang *et al*. (2009) attempted such a measurement for three saline lakes in Tibet, on the basis of *pufLM* sequences of the RC complex. By this measure, AAP relative abundance was similar to that in marine environments (0.95 to 5.35% of all bacteria) (Jiang *et al*. 2009). Diversity decreased with increasing salinity and pH, an expected community response to stress, but lower overall diversity than marine values was perplexing because all three lakes were less saline than the ocean (Jiang *et al*. 2009). Hence, factors controlling AAP diversity are still poorly delineated. However, the phylogenetic similarity between AAP assemblages from inland hypersaline systems (Yurkova *et al*., 2002; Csotonyi *et* al., 2008) and those from oceans implies that physico-chemical parameters outweigh geography in shaping AAP community structure.

1.4.2.3. Hypersaline springs.

Most hypersaline environments differ fundamentally from hydrothermal habitats on the basis of water flow. Because hydrothermal systems arise from geothermally heated water, all examples constitute springs. By contrast, hypersaline ecosystems are usually formed from desiccation and/or leaching of sediments, and are therefore generally more stagnant. Hypersaline springs are comparatively rare, and their microbiology is consequently poorly understood. Prior to this study, anoxygenic communities have scarcely been explored in such environments. Sulfide rich sediments of Salinas de Oro pond (2 to 10% salinity) resulting from a spring in the Spanish Pyrenees yielded the halotolerant (1 to 10% NaCl) vibrioid purple nonsulfur bacterium *Roseospira navarrensis* (Guyoneaud *et al*., 2002). However, AAP had never been isolated from saline springs (Yurkov and Csotonyi, 2003). As a result, exploration of the anoxygenic photosynthetic community of EGC (Chapter 2) contributes significantly to the understanding of

these unusual environments. The orange iron oxyhydroxide-rich microbial mats of the location (Fig. 1.2) are dominated by diatoms and the green algae *Percursaria percursa* (Londry *et al*., 2005; Csotonyi *et al*., 2008). A subterranean sulfate reducing community generates sulfide that supports anoxygenic phototrophs including relatives of *Roseospira* (similar to Salinas de Oro; Guyoneaud *et al*., 2002) and *Halochromatium* (Csotonyi *et al*., 2008). EGC springs possess an unusual overlap of the steep salinity and chemical gradients characteristic of meromictic lakes and the flowing water of rivers. Therefore, like the Bol'shaya thermal springs in which non-marine AAP were first discovered (Yurkov and Gorlenko, 1990), the EGC springs are an appropriate habitat in which to sample for both aerobic and anaerobic phototrophs (see Chapter 4).

Extreme environments are clearly important for understanding the taxonomic and physiological diversity of aerobic anoxygenic photosynthetic organisms. They also select for the evolution of unusual metabolic strategies, such as the transformation of toxic metal(loid)s, to which AAP exhibit exceptional resistance. A relevant background to metal(loid) resistance and transformation must first be laid.

1.5. Microbial metal(loid) resistance and transformation

Heavy metal(loid) resistance in bacteria is addressed by the fields of biogeochemistry, microbial ecology, enzymology, bioremediation and biotechnology. Research into microbe-metal interactions will undoubtedly increase as metal pollution rises in our increasingly industrialized societies because microorganisms possess enormous potential for use in metal(loid) recovery and bioremediation. Microorganisms express resistance to a diversity of metals, but discussion will be limited to the metalloids Se and Te, and the metal V. These elements have received

comparatively little attention. However, they occur at exceptionally high concentration in some extreme environments (see Section 1.5.1.). Furthermore, Te is increasing in occurrence as a consequence of elevated use in the electronics industry (Greer and Mathur, 2005).

1.5.1. Environmental distribution and chemistry of Te, Se and V

All three of the elements Te, Se and V are relatively rare on earth. Te ranks 75th in abundance in the earth's crust (Taylor, 1999), or 10^{-5} to 10^{-2} ppm in the lithosphere (Cox, 1989; Wray, 1998) and 5-17 x 10^{-8} ppm in the ocean (Lee and Edmond, 1985). Locally, however, it may be elevated by six to nine orders of magnitude, especially in association with gold, silver, iron, sulfur and copper (up to 14.8 ppm in mine tailings) (Oberthür and Weiser, 2008; Wray, 1998), and in hydrothermal systems (up to 17 ppm in deep ocean sulfides and 30.6 ppT in geothermal pipelines) (Knott *et al.*, 1995; Reyes *et al.*, 2002). Selenium is 10 to 10^4 times more common than Te: 0.05 to 0.14 ppm in the lithosphere (Cox, 1989), and 1.9 x 10^{-4} ppm in the ocean (Von Damm *et al.*, 1985). Like Te, it is more common in metal sulfides and hydrothermal systems (e.g. 213 to 1640 ppm) (Rouxel *et al.*, 2004). Terrestrially, Se is concentrated in soils influenced by weathering of Se-rich shales (Stolz and Oremland, 1999). Toxic levels of Se (up to 200 ppm) can accumulate in nearby hypersaline evaporation ponds, as in the San Joaquin Valley of California (de Souza *et al.*, 2001). Vanadium is the most abundant of the three metal(loid)s under discussion: 250 ppm in the lithosphere and 1.6 x 10^{-3} ppm in seawater (Cox, 1989). It is commonest in iron-enriched hydrothermal vent plume particles, and in oil reservoirs, where it reaches 2000 ppm (Macías-Zamora *et al.*, 1999). V therefore poses a serious threat as a pollutant in wastewater from petroleum mining and processing operations (Hernández *et al.*, 1998). Interestingly, some ascidians (subphylum *Urochordata*) accumulate V in their blood at 17,830

ppm (10^7 times background concentrations), employing it for microaerophilic oxygen transport or as an antifouling or antimicrobial agent (Michibata *et al.*, 2003).

Like sulfur, the chalcogens Se and Te are Group 16 elements, tending to form complex covalent compounds and possessing variable oxidation states: -II (methylated and metal tellurides or selenides), 0 (solid elemental form), +IV (tellurite or selenite, TeO_3^{2-} or SeO_3^{2-}) and most commonly +VI (tellurate or selenate, TeO_4^{2-} or SeO_4^{2-}) (Lee and Edmond, 1985). Se is incorporated into some essential biomolecules, such as selenomethionine and selenocysteine, but Te has no known biological requirement (Zannoni *et al.*, 2008). Nevertheless, it can enter cells of *Rhodobacter* via acetate permease (Borghese and Zannoni, 2010). Oxyanions of Te are about 100 to 1000 times more toxic than those of Se (Zannoni *et al.*, 2008), owing much of their potency to a strong oxidizing nature. They generate reactive oxygen species and free radicals that overwhelm the thiol-based antioxidant defense of bacteria (Turner *et al.*, 2001). A partially filled d-orbital explains the complex chemistry and variable oxidation states of V, from 0 (elemental form), to +II (V^{2+}), +III (V^{3+}), +IV (vanadyl, VO^{2+}) and +V (meta- or ortho-vanadate, VO_3^- or VO_4^{3-}) (Nies., 1999). V can replace Mb in the nitrogenase of *Azotobacter chroococcum* (Nies., 1999), but like Se, its compounds are toxic at high concentrations. Vanadate interferes with iron uptake by binding with siderophores (Baysse *et al.*, 2000) and exerts oxidative stress by forming reactive oxygen species (Aureliano *et al.*, 2002). Because vanadate resembles phosphate, it can enter cells through their phosphate-uptake systems and subsequently interfere with the function of ATPase (Nies, 1999).

1.5.2. Mechanisms of resistance to and reduction of Te, Se and V

Diverse microorganisms possess enzymatic mechanisms for resistance to and transformation of Te-, Se- and V-containing compounds, especially in environments enriched in them (Zannoni *et al*., 2008). Despite low global abundance of these elements, resistance is broadly distributed among anaerobic and aerobic phototrophs (Borsetti *et al*., 2009), hydrothermal vent heterotrophs (Rathgeber *et al*., 2002), halophilic microorganisms (Oremland and Stolz, 2000) and soil microbiota (Bautista and Alexander, 1972).

The wide taxonomic and phylogenetic distribution of microbial metal(loid) resistance may result from two processes. First, the high degree of sequence conservation among resistance determinants argues for their antiquity, perhaps representing relicts from survival strategies in metal-rich hot temperature environments where life first evolved (Agranov and Krishna, 1998). Alternatively, lateral gene transfer and/or plasmid exchange can explain the distribution of metal(loid) resistance: plasmids encoding it are mobilized and exchanged at interfaces between the marine environment and metal-enriched wastewater (Lebaron *et al*., 1994).

Mechanisms mediating microbial metal(loid) resistance to Te, Se and V include (1) reduced uptake, (2) increased efflux by ATP-driven pumps and chemiosmotic ion/proton antiporters, (3) metal-binding by metallothionein proteins, (4) biomethylation and (5) redox systems, which reduce toxicity by changing the oxidation state (Agranov and Krishna, 1998; Silver and Phung, 1996; Zannoni *et al*., 2008). Only the fifth strategy will be discussed here since even redox systems display great diversity, encompassing aerobic and anaerobic reduction, and biooxidation. These reactions may significantly influence biogeochemical cycling (Juniper and Tebo, 1995) and have important applications in bioremediation (Gadd, 2000). Biooxidation studies are scarce and this metabolic mode has only been reported for Se, never for Te nor V

(Ehrlich, 2002). Habitats most likely to host biooxidation of Te and V are magmatic deposits or gold and silver telluride ores (Oberthür and Weiser, 2008).

1.5.2.1. Aerobic Reduction

Aerobic mechanisms of Te^{IV} and Te^{VI} reduction are diverse, but still incompletely understood. At least seven unrelated genetic determinants confer resistance to tellurite (Zannoni *et al*., 2008). Most mediate a reduction reaction that accumulates deposits of black elemental Te in the cytoplasm, plasma membrane or periplasm (Zannoni *et al*., 2008). The diversity of Te resistance determinants has led some to propose that tellurite resistance and reduction are secondary effects of enzymes with other primary metabolic roles. In *Escerichia coli*, *R. sphaeroides* and a number of denitrifiers, periplasmic and membrane-bound nitrate reductases can reduce small amounts of tellurite (Avazéri *et al*., 1997; Sabaty *et al*., 2001). The affinity of tellurite for thiols allows cysteine residues to bind tellurite, mediating high-level tellurite reduction in *E. coli* (Zannoni *et al*., 2008). In some species electrons are sequestered from cytochromes to reduce tellurite (Moore and Kaplan, 1992; Trutko *et al*., 2000). Amino acid sequences of other Te resistance determinants resemble proteins with primary roles in S metabolism, modulation of intracellular toxic organic metabolite accumulation and heat shock response (Zannoni *et al*., 2008).

In contrast with Te, aerobic Se oxyanion reduction is somewhat scarce, possibly because of its relatively low toxicity compared to Te oxyanions. As for Te, nitrate reductases catalyze low level selenite reduction (Sabaty et al., 2001), and dark aerobic cultures of *R. sphaeroides* reduce large quantities of selenite to red Se^0 (Moore and Kaplan, 1992). A proteome analysis

suggested that an enzyme related to xenobiotic and morphinone reductases is involved in the reduction of selenite in *P. aeruginosa* (Bebien *et al*., 2001).

Aerobic microbial reduction of V oxyanions is even more rarely reported, probably because of its still lower toxicity than Te and Se, or because V bioreduction is preferentially an anaerobic process. The cyanobacteria *Nostoc puncteforme* and *Phormidium laminosum* accumulate V at concentrations up to 0.43 g/l (Lyalikova and Yurkova, 1992), while *Enterobacter cloacae* and *Escherichia hermannii* from petroleum contaminated soils accumulate 918 nmol of V per mg dry weight (Hernández *et al*., 1998). *Thiobacillus thiooxidans* reduces vanadate nonenzymatically via reduced sulfur metabolites (Ehrlich, 2002). Flavoenzymes such as glutathione reductase, lipoyl dehydrogenase and ferredoxin-NADP+ oxidoreductase can reduce V^V to V^{IV} with NAD(P)H (Shi and Dalal, 1993).

1.5.2.2. Anaerobic Reduction

Aerobically, microorganisms reap little benefit from oxyanions of Te, Se and V. Hence, aerobic toxicity of these compounds generally outweighs their utility. Anaerobically, however, low environmental redox potential raises the opportunity for exploitation of metal(loid)s as terminal electron acceptors in anaerobic respiration. Few chemical species outperform O_2 as a terminal acceptor of electrons from respiratory transport chains. However, in its absence, microorganisms utilize the most electrophilic alternatives, including oxidized nonmetals (e.g. NO_3^-, SO_4^{2-}) or metals (e.g. Fe^{3+}). Under these conditions, organisms that can harness Te, Se or V gain a competitive advantage because, for example, the reduction potential of the TeO_3^{2-}/Te couple (0.827 V) exceeds that of the SO_4^{2-}/HS$^-$ couple (-0.217 V) (Lloyd *et al*., 2001). Anaerobically, the greatest obstacle to utilizing these metal(loid)s is scarcity rather than toxicity.

Not surprisingly, the order in which respiratory use of these elements was discovered relates to global abundance and the familiarity of scientists with them: V and Se first, followed by Te.

Although anaerobic vanadate reduction is rarely reported, five bacteria are known to respire on vanadate: *Pseudomonas isachenkovii*, *Pseudomonas vanadiumreductans*, *Shewanella oneidensis*, *Geobacter metallireducens* and a relative of *Vibrio pomeroyi* (strain AV-V-25) (Csotonyi *et al*., 2006; Lyalikova and Yurkova, 1992; Rehder, 2008). The first two species were isolated from V-enriched ascidian blood and industrial wastewaters, respectively (Lyalikova and Yurkova, 1992). No V-specific reductases are known, but organisms enlist existing electron transport chains for vanadate respiration. *Pseudomonas* utilizes a membrane-bound nitrate reductase that favors nitrate over vanadate (Ehrlich, 2002), whereas *S. oneidensis* employs the outer membrane-bound polyheme *c*-type cytochrome OmcB, which also facilitates iron reduction (Rehder, 2008).

Some microorganisms, such as *Rhizobium* sp. strain B1 and sulfate reducers like *D. desulfuricans*, can reduce micromolar to millimolar concentrations of selenate to Se^0 or Se^{2-} without coupling it to growth (Hunter and Kuykendall, 2007; Lloyd *et al*., 2001). However, most research addresses the ecology, enzymology and bioremediative applications of anaerobic respiration on selenate and selenite (Oremland and Stolz, 2000). An understanding of its enzymology is incomplete, but as for dissimilatory reduction of vanadate, selenate respiration has clear ties with denitrification. *Sulfurospirillum barnesii* reduces both selenate and nitrate using the same constitutive reductase (Oremland *et al*., 1999). Even selenate-specific enzymes (e.g. SerA from *Thauera selenatis*) bear structural resemblance to nitrate reductase, suggesting a common evolutionary origin (McEwan *et al*., 2002). However, anaerobic reduction of selenite and selenate appear to be independent mechanisms, because (1) impairment of selenate reduction

does not affect selenite reducing activity in *E. coli* (Bébien *et al*., 2002), (2) *T. selenatis* respires on selenate and selenite using different enzymes (Schröder *et al*., 1997), and (3) selenite- and selenate-respiration usually occur in different species (Stolz and Oremland, 1999; Switzer Blum *et al*., 2001). Exceptions are the archaeon *Pyrobaculum aerophilum* (Huber *et al*., 2000) and a member of the *Aquificales* (Takai *et al*., 2002), which can respire on both oxyanions.

Until the discovery of respiration on tellurate by a relative of *Shewanella frigidimarina* from deep ocean hydrothermal vents (Csotonyi *et al*., 2006) (see Chapter 5), only non-respiratory anaerobic reduction of Te was known, such as mediated by cyt c_3 and hydrogenase in the sulfate reducer *Desulfovibrio desulfuricans* (Lloyd *et al*., 2001). Since then, increased experimental effort has yielded a new species that respires on tellurite in addition to tellurate (Baesman *et al*., 2009). Still, the study of anaerobic Te oxyanion reduction is in its infancy, and its enzymology has barely begun to receive attention. Thus far, no specific enzyme for tellurate or tellurite respiration has been isolated. An enzyme from *Bacillus* sp. STG-83 capable of anaerobic tellurite reduction is distinct from a selenite reducing enzyme of the same strain (Etezad *et al*., 2009). Similarly, Te^0 and Se^0 accumulation in different parts of *Shewanella oneidensis* indicates that distinct mechanisms are invoked for the reduction of each metalloid (Klonowska *et al*., 2005).

1.6. Metal(loid) reduction by prokaryotes from extreme environments

Although metal enriched habitats are extreme environments in their own right (Nies, 2000), some microorganisms must simultaneously endure additional extremes. For example, *Pyrobaculum arsenaticum* is a hyperthermophile that respires on selenate and arsenate at 95°C (Huber *et al*., 2000). Research is now turning its attention to extremophiles that are also 'metalophiles' or 'metalotolerant' and can detoxify metal(loid) oxyanions under these

conditions. Today, over 20 species from adverse environments can reduce oxidized Te and Se (Yurkov and Csotonyi, 2003). V transformation by extremophiles remains unpublished, although reduction of oxidized V at high temperatures is energetically favorable (Amend and Shock, 2001) and should support dissimilatory reduction. Extremophilic biotransformations of Se and Te display two important trends. First, hydrothermal vents and hypersaline systems contain a wealth of highly resistant microorganisms (Sections 1.6.1. and 1.6.2.). Second, extremotolerant AAP express the highest levels of resistance and reduction of tellurite among bacteria (Section 1.7.2.).

1.6.1. Aerobic and anaerobic metal(loid) reduction from hydrothermal vents

Deep ocean black smoker chimneys generate local extremes in temperature, pH, salinity and metal(loid) concentration (Kelley *et al*., 2002). This combination of stresses selects for microorganisms that tolerate several extremes simultaneously. Recently, *Pseudoalteromonas telluritireducens* and *Pseudoalteromonas spiralis* were described from samples of water, rocks and mat-like formations near Juan de Fuca Ridge black smokers (Rathgeber *et al*., 2002; 2006). Among the most metalloid-resistant bacteria known, the strains had MIC (minimum inhibitory concentration) of K_2TeO_3 and Na_2SeO_3 up to at least 2500 and 7000 µg/ml, respectively (the maximum tested, limited by solubility) and accumulated Te^0 and Se^0 as intra- and extracellular granules. These obligate aerobes were highly tolerant to extremes in temperature (5 to 45°C), pH (5 to 11) and salinity (0 to 20% NaCl), reflecting steep local gradients in these factors near black smoker chimneys (Kelley *et al*., 2002; Rathgeber *et al*., 2002; 2006).

Microorganisms that resist and reduce tellurite and selenite may have important biogeochemical and ecological roles in deep ocean hydrothermal vent ecosystems. High relative

abundance (at least 2% and 24% of all resident bacteria, respectively; Rathgeber *et al*., 2002)

implicates them as potentially important players in mineralization of sulfide chimney structures,

similar to species that precipitate iron, silver, copper and arsenic (Juniper and Tebo, 1995).

Because of the toxicity of oxidized Se and Te to other organisms, such biotransformation could

significantly modulate microbial and macrofaunal community development. For example,

symbiotic associations between alvinellid worms and metal(loid)-reducers may enhance the

metal-tolerance of these metazoans (Prieur *et al*., 1990), while the host's tissues would provide

the bacteria with a physically, chemically and nutritionally stable habitat. Relatives of

Shewanella frigidimarina and *Vibrio pomeroyi* on the surface of *Paralvinella sulfincola* worms

constituted the first report of dissimilatory reduction of tellurate, selenite and metavanadate at

hydrothermal vents (Csotonyi *et al*., 2006) (see Chapter 5). Since then, *Nautilia nitratireducens*,

a species capable of anaerobic respiration on selenate, has been isolated from sulfide structures

(Pérez-Rodríguez *et al*., 2010). Representing the class *Epsilonproteobacteria*, abundant primary

producers at hydrothermal vents, *N. nitratireducens* is multiply extremotolerant, growing at 25 to

65°C (optimum 55°C) and pH 4.5 to 8.5 (Pérez-Rodríguez *et al*., 2010).

1.6.2. Metal(loid) reduction from hypersaline environments

Hypersaline environments are sometimes enriched in metal(loid)s (Javor, 1989). Trace

elements are concentrated during evaporation of runoff from precipitation, irrigation or spring

water, leading to hypersaline pools with toxic levels of metal(loid)s (Ong *et al*., 1997). The

resident microorganisms, which are extensively adapted to regulating the balance between

intracellular and extracellular ion concentrations, may be preadapted to surviving in the presence

of concentrated metal(loid) oxyanions as well. In fact, hypersaline systems boast the highest

microbial metal(loid) resistance ever recorded. For the haloalkaliphilic archaea *Natronococcus occultus*, *Natronobacterium magadii* and *Natronobacterium gregoryi* from an alkaline lake, the MIC of potassium tellurite is an astounding 10 to 20 mM (2570 to 5140 µg/ml) (Pearion and Jablonski, 1999). This is only possible because of the high solubility of tellurite in alkaline media. Hypersaline Mono Lake in California is also the source of the newly described tellurite respiring halophile *Bacillus beveridgei* (optimum 0.5 M to 1.5 M NaCl, with growth up to 4 M) (Baesman *et al.*, 2009). Using hydrogen or lactate as electron donor, *B. beveridgei* can reduce 5 mM (1280 µg/ml) tellurite within 20 days. This gram-positive organism is phylogenetically distant from the gammaproteobacterial *Shewanella* relative representing the only other known example of dissimilatory Te oxyanion reduction (Csotonyi *et al.*, 2006). Environmental selection pressure outweighs phylogeny for this metabolic mode.

B. beveridgei* can also respire anaerobically on selenite (5 mM, or 860 µg/ml) (Baesman *et al.*, 2009), as can *Bacillus arsenicoselenatis*, *Bacillus selenitireducens* (both from Mono Lake; Switzer Blum *et al.*, 1998) and *Selenihalanaerobacter schriftii* (from the meromictic Dead Sea; Switzer Blum *et al.*, 2001). However, the highest levels of selenate resistance are found aerobically in a phylogenetically diverse assemblage (α- and γ-*Proteobacteria*, *Actinobacteria* and Gram-positive bacteria) from evaporation ponds in California's San Joaquin Valley where Se concentrations are 5 µg/ml in the water column and 200 mg/kg in evaporite deposits (de Souza *et al.*, 2001). Strain MPD-51 tolerates 5 M NaCl and 2 M selenate, volatilizing the oxyanion at a rate of 1.65 µg of Se/(g protein)/h. This is the most selenate-resistant organism ever reported.

1.6.3. Industrial and evolutionary significance of metal(loid) transformations

What is so attractive about metal(loid) transforming microorganisms? Evolutionarily, metal(loid) reducers (especially of iron) may have been among the earliest inhabitants of Earth (Lovley, 2002). Perhaps other metal(loid) transport systems developed concurrently, and ultimately gave rise to some of today's ion transport systems (Agranov and Krishna, 1998).

From an industrial standpoint, increasing cost and decreasing efficiency of abiological metal recovery from ores encourages the exploration of biologically mediated techniques of microbiometallurgy (Rawlings and Silver, 1995). Biooxidation and bioreduction serve important roles in metal(loid) recovery. Biooxidation, by a process called leaching, employs acidophilic chemolithotrophic Fe- and S-oxidizing bacteria, such as *Thiobacillus ferrooxidans* and *T. thiooxidans*, to disrupt the pyrite (FeS_2) matrix of an ore trapping the target metal, since biooxidation of pyrite can proceed 10^6 times faster than abiotic oxidation (Brierley and Brierley, 2002). The Fe^{3+} and acid incidentally generated also solubilize Te and V (Krebs *et al*., 1997).

Biometallurgy of trace metal(loid)s goes hand in hand with bioremediation, for biooxidation generates troublesome acidic and metalliferous tailings, which pollute surrounding ecosystems (Wray, 1998). Bioreduction precipitates these toxic elements from the water column for subsequent recovery. Commonly proposed microorganisms for this task are sulfate-reducers, which precipitate metals by raising pH and generating highly reduced sulfide (Hard *et al*., 1997). Some sulfate reducers also enzymatically reduce selenite, selenate and tellurite, but only weakly (Lloyd *et al*., 2001).

Extremotolerant metal(loid) reducers may provide the best solution for bioremediation. Enzymatic reduction accumulates metal(loid)s in elemental form, requiring little or no further purification, unlike metal sulfides precipitated by sulfate reducing bacteria. Species from

extreme habitats frequently display multiple extremotolerance, making them useful in diverse

industrial settings. They exhibit the most intense metal(loid) reduction because their native

habitats are enriched in these elements. *Pseudoalteromonas* from hydrothermal vents grows at

pH 5 to 11 and rapidly reduces 9.9 and 40.5 mM tellurite and selenite, respectively (Rathgeber *et

al*., 2002; 2006). Interestingly, some heterotrophs such as *Pseudomonas* can couple selenate or

tellurite reduction to oxidation of organic toxins such as benzene derivatives (Knight *et al*.,

2002), toluene (Sanchez-Romero *et al*., 1998) or polychlorinated biphenyls (Zanaroli *et al*.,

2002). The oligotrophic nutrition of highly metal(loid) resistant anoxygenic phototrophs

(especially AAP) is attractive because energy requirements are supplemented with light (Yurkov

and Csotonyi, 2009), decreasing the projected cost of bioremediative application. Autotrophs

such as selenate-respiring *P. aerophilum* (Huber *et al*., 2000), vanadate-respiring *P. isachenkovii*

and *P. vanadiumreductans* (Lyalikova and Yurkova, 1992) are also appealing because they need

no organic amendment.

Employing microbial consortia further increases efficiency of microbioremediation by

mimicking natural community function, each species mediating a different step in biochemical

transformation. For example, co-cultures of *B. arsenicoselenatis* (reducing selenate to selenite)

and *B. selenitireducens* (reducing selenite to Se^0) achieve complete selenate reduction (Switzer

Blum *et al*., 1998). Artificially constructed microbial mats of cyanobacteria, purple phototrophs

and other microorganisms immobilized within silica particles can reduce Cr^{VI} to Cr^{III} and Se^{IV} or

Se^{VI} to Se^0, while degrading organic pollutants such as trinitrotoluene (TNT) (Pümpel and

Paknikar, 2001). A similar system might involve AAP, given their extreme metal(loid) resistance

(Section 1.7.), frequent extremotolerance (Section 1.4.) and common natural association with

cyanobacterial mats (Yurkov and Csotonyi, 2009).

1.7. Interaction of metal(loid) resistance and photosynthesis

Both photosynthesis and redox-based metal(loid) transformations (such as anaerobic respiration or aerobic reduction) rely on controlled transport of electrons down energetic gradients. In anoxygenic photosynthesis, electrons from photo-oxidized BChl are funneled through an electron carrier system to harness their energy before returning to the RC to re-reduce BChl (Madigan *et al.*, 2003). The mechanism of aerobic Te oxyanion reduction is less clear, but anaerobic respiration likewise involves electron shuttling along a respiratory chain, terminating at the metal(loid). In some instances, organisms possess both photosynthetic and metal(loid) reduction systems. For example, purple nonsulfur bacteria such as *R. sphaeroides* non-respiratorily reduce tellurite and selenite during photosynthesis (Moore and Kaplan, 1992; 1994). The acidophilic AAP *Acidiphilium acidophilum* can couple ferric iron reduction to glucose oxidation microaerophilically (Marchand and Silverstein, 2003). Other AAP from extreme environments (e.g. *E. ursincola* and *E. ramosum*) exhibit the highest aerobic resistance to (and reduction of) tellurite known among bacteria (Yurkov *et al.*, 1996). The electrochemical basis of these metabolic modes and the presence of both systems in a single species make it reasonable to expect interactions to occur between photosynthesis and metal(loid) reduction. In light of this potential connection, surprisingly little study has been invested into the field, most of it accomplished over a decade ago.

1.7.1. Metal(loid)s for disposal of excess reducing power

One body of research addressed the use of metalloid oxyanions for disposal of excess reducing power generated during photosynthesis by purple nonsulfur bacteria such as *R.*

sphaeroides, R. capsulatus, R. palustris and *Rhodopseudomonas viridis* (Moore and Kaplan, 1992). Although these species cannot utilize Te and Se oxyanions as terminal electron acceptors, *R. sphaeroides* displays high level resistance to selenite (800 μg/ml), selenate (150 μg/ml), tellurite (600 μg/ml) and tellurate (500 μg/ml). Anaerobic reduction requires both an intact CO_2 fixation pathway and a functional photosynthetic electron transport chain, and is inversely related to the oxidation state of the photoheterotrophically utilized carbon source (Moore and Kaplan, 1994). This implies that metal(loid)s help to maintain redox poise by providing a sink for reducing equivalents that build up during active photosynthesis, thereby preventing the arrest of the process. It is unknown whether the more remarkably high level tellurite reduction (1524 μg/ml) in two marine purple nonsulfur strains (NKPB160041 and NKPB030619) serves a similar purpose (Yamada *et al*., 1997).

1.7.2. High level metal(loid) resistance among AAP

Although anaerobic anoxygenic phototrophs are highly metal(loid) resistant, AAP display the greatest tellurite resistance and reduction known among bacteria. Seven species (*E. hydrolyticum*, *E. ramosum*, *E. ezovicum*, *E. ursincola*, *E. litoralis*, *R. thiosulfatophilus* and *S. sibiricus*) survive K_2TeO_3 at 1200 μg/ml, and three species grow with 2300 to 2700 μg/ml (Yurkov *et al*., 1996). Most strains reduce tellurite to Te^0, sometimes generating deposits over 0.5 μm long that occupy 20 to 30% of the cell volume (Yurkov and Beatty, 1998a).

Although *Hoeflea phototrophica* (which tolerates 1000 μg/ml of K_2TeO_3) comes from a marine dinoflagellate culture (Biebl *et al*., 2006), all other metal(loid) resistant AAP were isolated from extreme environments. Six of the original seven investigated organisms hail from thermal (and often alkaline) spring systems (Yurkov and Beatty, 1998a), while *E. litoralis* was

isolated from a hypersaline marine microbial mat (Yurkov and van Gemerden, 1993a). Selenite-, selenate-, tellurite-, arsenate- and vanadate-resistant *C. bathyomarinum* is of hydrothermal vent plume origin (Yurkov *et al.*, 1998b; Yurkov and Csotonyi, 2003). Eight of fifteen strains from the EGC hypersaline springs grow with 1000 μg/ml of tellurite, selenite and metavanadate, and all fifteen strains resist at least one of the metal(loid)s (Csotonyi and Yurkov, unpublished). Although high correlation between physical stress and metal(loid) resistance in AAP follows from the fact that extreme environments often display high metal(loid) concentrations, firm conclusions will require a broader survey of species from ordinary environments as well.

Like purple nonsulfur bacteria (More and Kaplan, 1992; Sabaty *et al.*, 1998), AAP may utilize oxidized Te to balance intracellular redox poise during active growth. Results indicate that an analogous process involving re-oxidation of electron carriers such as NADH, FADH$_2$ or quinones reduced during respiration rather than photosynthesis might occur aerobically in AAP such as *R. thiosulfatophilus* (Yurkov *et al.*, 1996). This species even possessed a similar relationship between the extent of tellurite reduction and the oxidation state of its carbon source as in purple nonsulfur bacteria. However, the mechanism of tellurite reduction and resistance has not been sufficiently investigated in AAP. The markedly higher tellurite resistance of this group than that of purple nonsulfur bacteria suggests that a different, or at least additional, mechanism may function in AAP. For example, they may possess novel metal(loid)-specific reductases. However, because their abundant suite of photosynthetically-decoupled carotenoids has been suggested to confer protection from photooxidative damage (Beatty, 2002), the photosynthetic apparatus or related pigment systems may also be involved in ameliorating metal(loid)-induced toxicity (see Chapter 7). Carotenoids possessing more than nine conjugated double bonds (e.g. zeaxanthin and erythroxanthin sulfate that constitute the majority of this class of pigments in

AAP such as *E. ramosum* and *E. litoralis*; Yurkov *et al*., 1993) are especially capable of quenching reactive oxygen species (El-Agamey and McGarvey, 2008). Investigation of the influence of metal(loid)s on the photosynthetic apparatus of phototrophs, especially AAP, is therefore warranted.

1.7.3. Influence of metal(loid)s on photosynthesis and photosynthetic pigments

The impacts of chalcogens such as Te and Se on photosynthetic pigment production have not been investigated at all. Focusing on the more toxic of the two, the effects of Te can only be speculated upon, based on its other biological activities and on the action of similar elements. Tellurite likely acts in two different ways on the photosynthetic apparatus: via oxidative stress and by mimicking iron deficiency.

Tellurite causes oxidative stress by the generation of reactive oxygen species during its attack on the cell's glutathione-based oxidative defense system (Zannoni *et al*., 2008). In purple nonsulfur bacterial mutants lacking photoprotective carotenoids, photooxidative stress induces mutations in photosynthesis genes. This leads to cells that accumulate intermediates in the biosynthesis of BChl, including protoporphyrin IX and chlorophyllide *a* (Ouchane *et al*., 1997). It follows that tellurite could also induce similar oxidative effects in phototrophs, but the hypothesis has never been tested. Because most bacteria are sensitive to K_2TeO_3 at concentrations above 1 µg/ml (Taylor, 1999), subject species for investigation must be chosen that are unusually resistant to the toxic oxyanion. AAP are the most competent in this regard.

The covalent radius of Te (138 pm) closely resembles that of Fe (132 pm) (Cordero *et al*., 2008), and therefore it may be hypothesized to substitute for Fe in enzymatic reactions in a similar way that Mn and other metals block the access of Fe in biological systems (Csatorday *et*

al., 1984). Exposure of the cyanobacterium *Anacystis nidulans* to Mn^{2+} induces accumulation of

Mg protoporphyrin (MgP), a precursor in the biosynthetic pathway of Chl (as well as BChl). The

authors attributed the phenomenon to blockage of a Fe-requiring step (Csatorday *et al.*, 1984). In

fact, in both plants and the purple nonsulfur bacterium *R. sphaeroides*, Fe is required for the

formation of MgP (Lascelles, 1966; Spiller *et al.*, 1982). Furthermore, the gene *acsF* in *R.

gelatinosus* that is responsible for the enzyme catalyzing cyclization of MgP monomethyl ester

encodes a binuclear-iron-cluster-containing protein (Pinta *et al.*, 2002). The Fe-replacement

effect of metals such as Mn^{2+} differs substantially from that of the toxic metal Co^{2+}, which in

both *A. nidulans* and *R. sphaeroides* inhibits Chl or BChl synthesis, but causes the accumulation

of protoporphyrin instead of MgP (Csatorday *et al.*, 1984; Giotta *et al.*, 2006). In this case,

inhibition is due to blockage of Mg uptake by the cell, preventing the chelation of protoporphyrin

to MgP. Spectrophotometric analysis of accumulated BChl biosynthesis intermediates therefore

allows distinction between some of the modes by which BChl inhibition occurs, because

protoporphyrin and MgP have distinct absorbance spectra.

Similarly scant information exists on the action of V (in the form of vanadate) on

photosynthesis, which is markedly different from that of most metals. Although its influence has

not been tested on anoxygenic phototrophs, vanadate actually surprisingly stimulates growth and

photosynthetic activity in the green algae *Chlorella fusca* and in spinach plants (Meisch and

Becker, 1981). At micromolar concentrations (20 μM), vandate brings about a 3-fold increase in

PS I activity, and is still stimulatory to photosynthetic O_2 production up to 0.1 mM (Meisch and

Becker, 1981). Vanadate is not irreversibly reduced in the process and the authors suggest that

the oxyanion acts as a redox catalyst between photosystems II and I. Given this intriguing effect,

it is surprising that no further work has followed up these preliminary results on the influence of

V compounds on photosynthesis. Anoxygenic phototrophs, unlike their oxygenic counterparts, possess only a single PS, a fact which precludes the suggested catalytic role of vanadate. However, the possibility of vanadate acting as an electron shuttle between other metabolic junctions, either photosynthetic or respiratory, should inspire investigation of the effect of vanadate on the highly metal(loid) resistant AAP (see Chapter 6).

1.8. Thesis objectives

Hydrothermal vents and hypersaline springs can provide key insights into the taxonomy and physiology of anoxygenic phototrophs and metal(loid) transforming microorganisms. The highly metal(loid) resistant AAP are especially prominent members of these environments. This project set out to elucidate the phylogenetic and physiological richness of unusual hydrothermal (Axial Volcano: 45°56′00″ N; 130°00′ 51″ W ; Explorer Ridge: 49°45′38″ N; 130°15′ 23″ W; Fig. 1.1) and hypersaline (East German Creek: 52°45′10″ N, 100°52′ 50″ W ; Fig. 1.2) systems in the context of anoxygenic photosynthesis and metal(loid) resistance. The specific objectives of this thesis are to:

(1) Enumerate the anoxygenic phototrophs and to quantify the numerical importance of AAP in a unique type of extreme environment, a spring system that combines the hypersalinity of usually static water bodies with the turbulent and therefore highly oxygenated conditions of flowing rivers (Chapter 2). The essentially plant-devoid EGC represents a structurally simplified community in which interactions of microorganisms are easier to evaluate than in more complex systems replete with macroorganisms, and therefore it provides an invaluable system in which to investigate the microbial ecology of extreme environments;

(2) Isolate from the EGC springs and taxonomically describe novel species and genera of anoxygenic phototrophs bearing special phylogenetic relevance (e.g. gammaproteobacterial AAP) or possessing previously unknown physiological characteristics relating to photosynthesis (flexible oxygen tolerance of anoxygenic photosynthesis) and metal(loid) metabolism (high metallotolerance or metallophily) (Chapters 3, 4, 6);

(3) Enrich for and characterize a previously unreported form of metalloid metabolism, anaerobic respiration using potassium tellurate as a sole terminal electron acceptor in bacteria from a deep ocean hydrothermal vent community (Chapter 5);

(4) Bridge the topics of anoxygenic photosynthesis and metal(loid) resistance by assessing the influence of metal(loid)s, especially tellurite, on photosynthetic pigment production in highly metal(loid)-resistant AAP isolated from hydrothermal and hypersaline habitats (Chapter 7).

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Chapter 2.

Novel halophilic aerobic anoxygenic phototrophs from

a Canadian hypersaline spring system.

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The first author was the major contributor to research presented.

2.1 Abstract

The first enumeration of cultivable obligately aerobic phototrophic bacteria from a terrestrial saline spring was accomplished in the East German Creek system (salinity ~6%), near Lake Winnipegosis, Manitoba, Canada. Occurring at densities up to 3.3×10^{7} CFU/ml of sample, aerobic phototrophs comprised 15-36% of the total cultivable bacterial population in the diatom- and chlorophyte-dominated aerobic microbial mats. Many of the representative strains isolated for phenotypic characterization and phylogenetic analysis possessed <96% 16S rDNA sequence overlap with published species, including an obligately aerobic phototrophic gammaproteobacterium displaying only 92.9% 16S rDNA sequence similarity to *Congregibacter litoralis*. The springs yielded the most highly halotolerant aerobic anoxygenic phototroph yet recorded, strain EG11, which grew with 26% NaCl.

Keywords: aerobic anoxygenic phototrophs; phototrophic halophiles; hypersaline springs; bacteriochlorophyll, physiology

2.2. Introduction

The globally abundant obligately aerobic anoxygenic phototrophs (AAP) (comprising up to 10% or more of microbes in illuminated environments; Rathgeber *et al.* 2004) differ drastically from conventional anoxygenic phototrophic bacteria in requiring oxygen for the assembly and functioning of their BChl *a*-based photosynthetic apparatus (Yurkov 2006). AAP augment chemoheterotrophic catabolism with phototrophic energy generation (Yurkov and Csotonyi 2003). Although they are incapable of photoautotrophy, their light-assisted chemoheterotrophy modulates nutrient turnover more efficiently than does strict chemoheterotrophy, since phototrophic energy production allows a greater fraction of their reduced carbon pool to be shunted to biomass production (Yurkov and Csotonyi 2009). Their large populations, efficient metabolism and ability to utilize solar energy in the extensive aerobic fraction of habitats make them key players in modulating resource availability in illuminated environments in which microbial primary production predominates (Yurkov and Csotonyi 2009). The relatively high resistance of AAP membrane potentials to extremes in temperature and pH (Jiao *et al.* 2004) suggests that AAP may be particularly competitive in extreme environments, from which more than half of their diversity has been described (Rathgeber *et al.* 2004; Yurkov and Csotonyi 2003). AAP might therefore exert greater proportional biogeochemical influence in such habitats than in more mesophilic environments because conditions are often prohibitive for substantial plant cover (McKillop *et al.* 1992). Halotolerant AAP were first isolated from tidal flats and pools (e.g. *Erythrobacter litoralis*, *Roseivivax halodurans*, *Roseivivax halotolerans*) (reviewed by Yurkov and Csotonyi 2003), then from lentic ecosystems such as soda and meromictic lakes (e.g. *Roseivarius*, *Roseinatronobacter, Roseisalinus,*

Roseicyclus) (reviewed by Yurkov and Csotonyi 2003, 2009). However, this study of a central Canadian saline spring system known as East German Creek (EGC) represents the first enumeration and investigation of physiology and phylogenetic affinity of AAP from saline lotic (stream) environments, which uniquely combine high salinity with flowing water.

In many terrestrial saline systems, cyanobacteria produce a scaffold, throughout aerobic regions of which AAP respire along with purple and green nonsulfur bacteria (PNSB and GNSB) (Yurkov 2006). At deeper levels in habitats supplied with reduced sulfur compounds, purple and green sulfur bacteria may form distinctly colored strata at sufficiently anoxic and often sulfide-rich depths which are still adequately illuminated (Pfennig and Trüper 1992; Trüper and Pfennig 1992). Although AAP have been isolated from marine dinoflagellates, the EGC springs represent the first terrestrial AAP-sampled saline mats dominated by eukaryotes (green algae and diatoms) instead of cyanobacteria (Londry *et al.* 2005). Hence, a novel suite of AAP could be encountered.

Palynological records (Patterson *et al.* 1997) suggested that the EGC springs have been active for at least 5500 years, releasing water laden with salts from subterranean ancient marine deposits (Grasby 2000). The constant terrestrial source of hypersaline water, precluding the need for connection with extraneous water bodies to ensure continuous hydration, should allow highly endemic phototrophic populations to develop. Previous studies of other saline springs have yielded taxonomically and morphologically novel forms of PNSB (Guyoneaud *et al.* 2002).

2.3. Materials and methods

2.3.1. Sampling and physico-chemical analysis

Measurements and sampling were conducted on 18 May 2002, at East German Creek (EGC) (52° 45′ 10″ N, 100° 52′ 50″ W), near Lake Winnipegosis, Manitoba (Site 16 of McKillop *et al.* 1992). Samples of water, sediment and microbial mat (1–2 cm^2) were transported on ice to the laboratory for analysis. Water pH was measured on site using a Beckman ϕ255 pH meter, and temperature with a Springfield Duo-Temp digital thermometer. Total dissolved solids (TDS) were determined in the laboratory by weighing 10-ml liquid samples before and after drying at 70°C for 24 hours.

2.3.2. Enumeration and cultivation

Bacterial populations were investigated by culturing on media designed for AAP and PNSB. Despite disregarding uncultivable members of the community, this technique allowed subsequent phenotypic investigation of isolated representatives. Although AAP were the focus of investigation, representative anaerobic phototrophs and non-phototrophs were also isolated and phylogenetically characterized. In the laboratory, mat samples were homogenized by vortexing 2-ml portions of suspensions in screw-capped tubes, serially diluted and inoculated onto agar-containing aerobic plates of rich organic Medium A for AAP and anaerobic agar deeps of Medium B (for PNSB) and Medium C (for purple sulfur bacteria). Previously described vitamin (VS) and trace element (TES) solutions were used (Yurkov 2006). Media were altered from published sources (Imhoff 2001, 2003; Yurkov 2006) to approximate the springs' mineral composition, and adjusted

to pH 7.0 with 0.5 M HCl or 0.5 M NaOH after autoclaving at pH 5.9. Medium A contained (g/l): MgSO$_4$·7H$_2$O, 3; Na$_2$SO$_4$, 1.8; KH$_2$PO$_4$, 0.3; NH$_4$Cl, 0.3; KCl, 0.7; CaCl$_2$·2H$_2$O, 0.05; NaCl, 40; Na-acetate·3H$_2$O, 1; Difco Yeast Extract, 1; Difco Bactopeptone, 0.5; Casamino Acids, 0.5; TES, 2 ml; VS, 2 ml. Medium B contained (g/l): MgCl$_2$·6H$_2$O, 2.5; Na$_2$SO$_4$, 3.5; KH$_2$PO$_4$, 0.3; NH$_4$Cl, 0.5; KCl, 0.7; CaCl$_2$·2H$_2$O, 0.05; NaCl, 40; Na-acetate·3H$_2$O, 1; Difco Yeast Extract, 0.5; NaHCO$_3$, 1.5; FeCl$_3$, 2.9x10^{-4}; Na$_2$S$_2$O$_3$·5H$_2$O, 0.3; TES, 10 ml; VS, 2 ml. Medium C contained (g/l): MgSO$_4$·7H$_2$O, 3; Na$_2$SO$_4$, 1.8; KH$_2$PO$_4$, 0.3; NH$_4$Cl, 0.5; KCl, 0.7; CaCl$_2$·2H$_2$O, 0.05; NaCl, 40; NaHCO$_3$, 1.5; Na$_2$S·9H$_2$O, 0.36; TES, 10 ml; VS, 2 ml.

All cultures were incubated at 28°C, AAP in continual darkness, and anaerobic anoxygenic phototrophs in a continuously incandescently illuminated (3000 lux), temperature-controlled Conviron incubator, model 125L. Colonies were counted and characterized by color and morphology after 7 days. Phototrophs were enumerated by measuring spectra of several subcultured representatives of each pigmented colony morphotype, and calculating the proportion that produced BChl (Yurkov and van Gemerden 1993). Phototrophs were thus differentiated from other pigmented strains.

2.3.3. 16S rDNA sequencing and phylogenetic analysis of isolates

Ribosomal DNA from 15 strains of AAP, 3 anaerobic anoxygenic phototrophs and 3 aerobic non-phototrophs was sequenced. Extraction of genomic DNA, PCR-mediated amplification of complete 16S rRNA gene sequences and direct sequencing of PCR products were carried out as by 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.* 1999), and similarity values were determined. A neighbour-joining dendrogram was reconstructed from a distance matrix using the treeing algorithm of Felsenstein (1993). Bootstrap values were determined according to Felsenstein (1985). Accession numbers obtained for strains are displayed in Fig. 2.2.

2.3.4. Phenotypic characterization of isolates

Cellular morphology of purified strains and of field samples was assessed with a Zeiss Axioscop 2 phase contrast microscope equipped with a DVC digital camera. Pigmented strains were screened spectrophotometrically (with a Hitachi U-2010 spectrophotometer) at 350–1100 nm for *in vivo* diagnostic absorption peaks of bacteriochlorophyll (BChl) (360–1020 nm), chlorophyll (Chl) (430–700 nm) and carotenoids (400–550 nm). Absorption spectra of overnight extracts (acetone/methanol, 7:2 *v/v*) of selected pure cultures were also obtained. Salinity (0-30% NaCl) and pH (4-12) tolerance profiles, and carbon source requirements (compounds listed in Table 2.3), were acquired at $28^{\circ}C$ in continual darkness as described (Yurkov *et al.* 1999). Capacity for anaerobic photosynthesis was assessed by scoring growth on both plate and liquid illuminated cultures, using Medium B amended with Na-malate (0.3 g/l) and Na-succinate (0.3 g/l) as additional carbon and electron sources, and with and without sulfide (0.36 g/l $Na_2S \cdot 9H_2O$) as electron donor for liquid cultures. Anaerobic photosynthesis was also assayed on modified CENMED medium designed for *Rhodocista centenaria* (Stadtwald-Demchick *et al.* 1990), containing (g/l): $MgSO_4 \cdot 6H_2O$, 0.2; KH_2PO_4, 0.6;

K$_2$HPO$_4$, 0.9; Na$_2$-EDTA, 0.005; NH$_4$Cl, 1; CaCl$_2$·2H$_2$O, 0.075; NaCl, 40; Na-pyruvate, 2.2; Difco Yeast Extract, 0.1; Na$_2$S$_2$O$_3$·5H$_2$O, 0.5; chelated iron solution, 2 ml; TES, 2 ml; VS, 2 ml.

2.4. Results and discussion

2.4.1. Study site

A large salt pan (~300 × 400 m) with few halotolerant plants (e.g. *Salicornia*; McKillop *et al.* 1992) was stained reddish-orange by extensive deposition of limonite (iron oxyhydroxide) (Fig. 2.1a). Widespread patches of white, indicating heavy salt precipitation, signified the extremely hypersaline conditions surrounding the pools and streams (Fig. 2.1b). Black subterranean sediments emitted the odor of hydrogen sulfide. Water discharging at up to 4800 l/h (McKillop *et al.* 1992) from crater-like springs (15–100 cm deep by up to 2 m wide, with shallow peripheral shelves 5 cm deep) (Fig. 2.1a) atop raised mounds of sinter generated shallow streams (Fig. 2.1c) tens or hundreds of meters long.

EGC was sampled at 5 sites (Fig. 2.1d). Sites 1 and 5 were springs lined with fine grey benthic sediments and (at Site 1) a large floating green microbial community. Sites 2 and 4 were in an outflow channel 5 m and 100 m downstream of its parent spring, respectively, the former bearing both a several-cm-thick orange and green floating microbial community (Fig. 2.1c) and a 1-mm-thin olive green and brown benthic mat, the latter possessing only the benthic mat. Site 3, 100 m downstream of a different spring, resembled Site 4, but had numerous stones and a more yellowish green benthic mat.
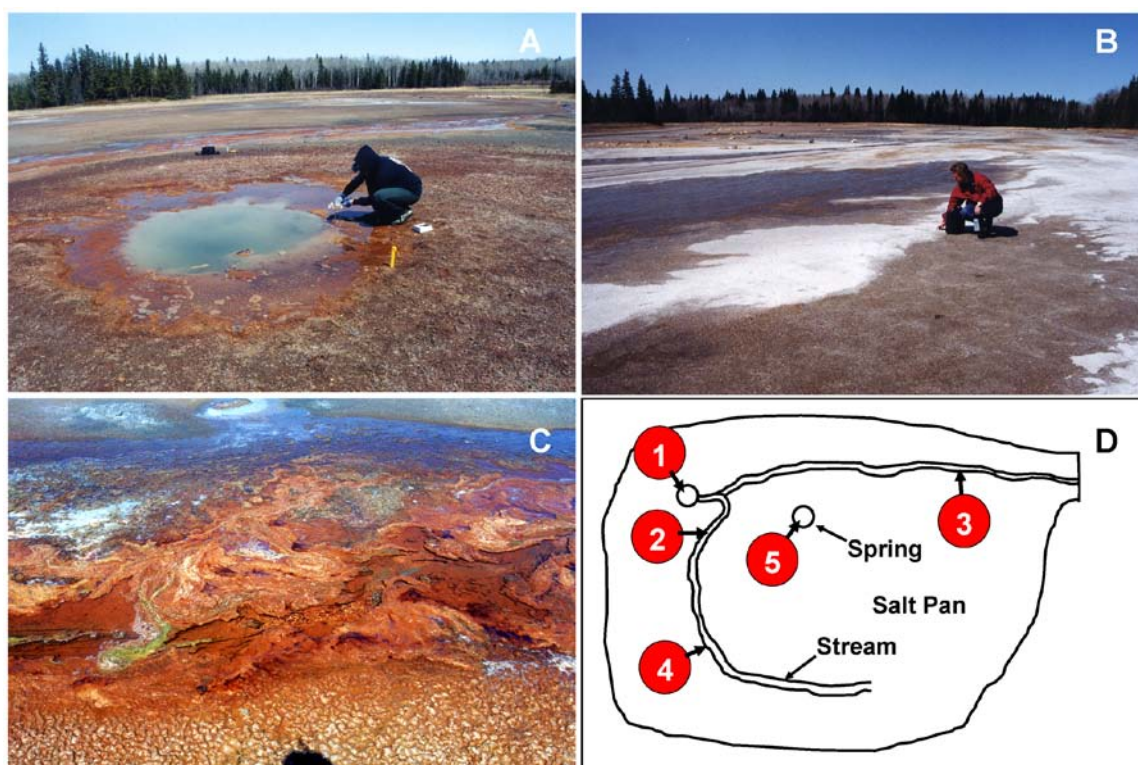
Fig. 2.1. East German Creek brine springs. **(a)** Spring pool, with effluent stream and limonite-stained salt pan in background. **(b)** White evaporate deposits of NaCl. **(c)** Effluent stream (Site 2) from main spring (Site 1), demonstrating a thick floating microbial mat. **(d)** Map of EGC to showing positions of sampling sites 1 to 5 (red dots).

2.4.2. Physico-chemical analysis

pH was approximately neutral, but rose by 0.7 units from springs to streams (Table 2.1), possibly due to loss of CO_2 upon atmospheric exposure (McKillop *et al.* 1992). TDS values (56.7–67.3‰, *w/v*) (Table 2.1) bracketed the value (61.6‰) reported by McKillop *et al.* (1992), with variation likely due to evaporation or dilution by intersection with subterranean fresh water conduits.

2.4.3. Microscopic survey of natural samples

Although AAP could not be distinguished microscopically, identifiable dominant organisms reflected the aerobic nature of the sampled microhabitats. Most of the biomass composing the thick floating mat-like growth at Site 2 was the North American/European filamentous coastal marine green alga *Percursaria percursa* (*Chlorophyta*: *Ulvaceae*), of which EGC and surrounding spring systems constitute the only interior North American account (Londry *et al.* 2005). Filamentous cyanobacteria were rarely observed (a few filaments in only 2 samples). Pennate diatoms dominated all benthic samples from Sites 1, 3 and 4 (diatoms were the only algae observed in Site 3), and co-dominated floating mats from Site 2 with *Percursaria*. Sulfur-like intracellular inclusions were present in rare filamentous *Thiothrix*-like cells from Sites 2 and 5, but sulfur-inclusion-filled short rods typical of purple sulfur bacteria were absent. These observations are consistent with the fact that none of the sample profiles possessed the purple color banding typical of anaerobic phototrophs in laminated mats.

Table 2.1. Physico-chemical properties of sampling stations at EGC.

Site	Description	Measurement Source	pH	TDS (‰, *w/v*)
1	Spring pool (2 x 1 m) [a]	pool center periphery	7.05 7.03	66.3 NA
2	Stream (30 x 5 cm) [a]	central channel	6.90	65.9
3	Stream, (30 x 5 cm) [a]	central channel	7.22	NA
4	Stream (30 x 5 cm) [a]	central channel	7.51	67.3
5	Spring pool (1.5 x 0.2 m) [a]	pool center periphery	6.80 7.08	56.7 NA

[a] measurements are given as width x depth. NA, not applicable.

2.4.4. Enumeration

About 15-32% of the 6.96–11.26 $\times$ 10^6 CFU/ml aerobic bacteria of Sites 1, 2 and 4 were anoxygenic phototrophs (Table 2.2), in accord with the 0.3-3.6 $\times$ 10^7 CFU/ml of AAP reported by Yurkov and van Gemerden (1993) for a Dutch supralittoral cyanobacterial microbial mat. Paucity of anoxygenic phototrophs in anaerobic EGC enumerations, and the fact that no PNSB were recovered from aerobic plates (even though the PNSB EG17 subsequently grew on Medium A), implies that AAP constituted the vast majority of this population, reflecting its O_2-rich algal environment. The AAP fraction was greatest (~36%) in the most diatom-rich Site 3 sample. This value markedly exceeds the 23% BChl-containing fraction (AAP and PNSB) of all surface bacteria cultivated on Na_2SO_4-amended (15 g/l) aerobic media in meromictic Mahoney Lake (Yurkova *et al.* 2002). Although diatom-dominated EGC mats are the first such communities screened for AAP, these observations suggest that eutrophic diatomaceous mats may be especially replete in AAP. By comparison, AAP usually form <10% of oligotrophic marine bacterial populations, with the highest relative abundance (up to 18.74%) in temperate nutrient-rich estuaries (reviewed by Yurkov and Csotonyi 2009).

2.4.5. Phylogenetic analysis of isolates

All sequenced strains from this cold continental hypersaline community were related to either marine (11 strains) or meromictic lake (10 strains) species (Fig. 2.2). Representative non-phototrophs and anaerobic anoxygenic phototrophs were sequenced to briefly describe the non-AAP community. Non-phototrophic bacteria included

Table 2.2. Enumeration of bacteria cultured from aerobic microhabitats at four of five

EGC sampling sites.

Culture Conditions and Organism Type	CFU/ml x 10^6 (and percent of total)			
	Site 1 thin brown pool mat	**Site 2** thick orange stream mat	**Site 3** thin yellow stream mat	**Site 4** thin brown stream mat
Aerobic Plates (Medium A)				
Pigmented	2.08 (29.9)	7.14 (63.4)	74.25 (81.4)	1.58 (20.6)
Anoxygenic phototrophs	1.09 (15.7)	2.37 (21.1)	33.18 (36.4)	2.48 (32.4)
Total Aerobic	6.96	11.26	91.25	7.66
Anaerobic Agar Deeps (Medium B)				
Total pigmented	0.01 (2.4)	0.02 (0.3)	0.16 (5.1)	0.35 (3.8)
Anoxygenic phototrophs	0 (0)	0.0002 (0.003)	0.04 (1.3)	0.0001 (0.001)
Total Anaerobic	0.41	5.72	3.16	9.15
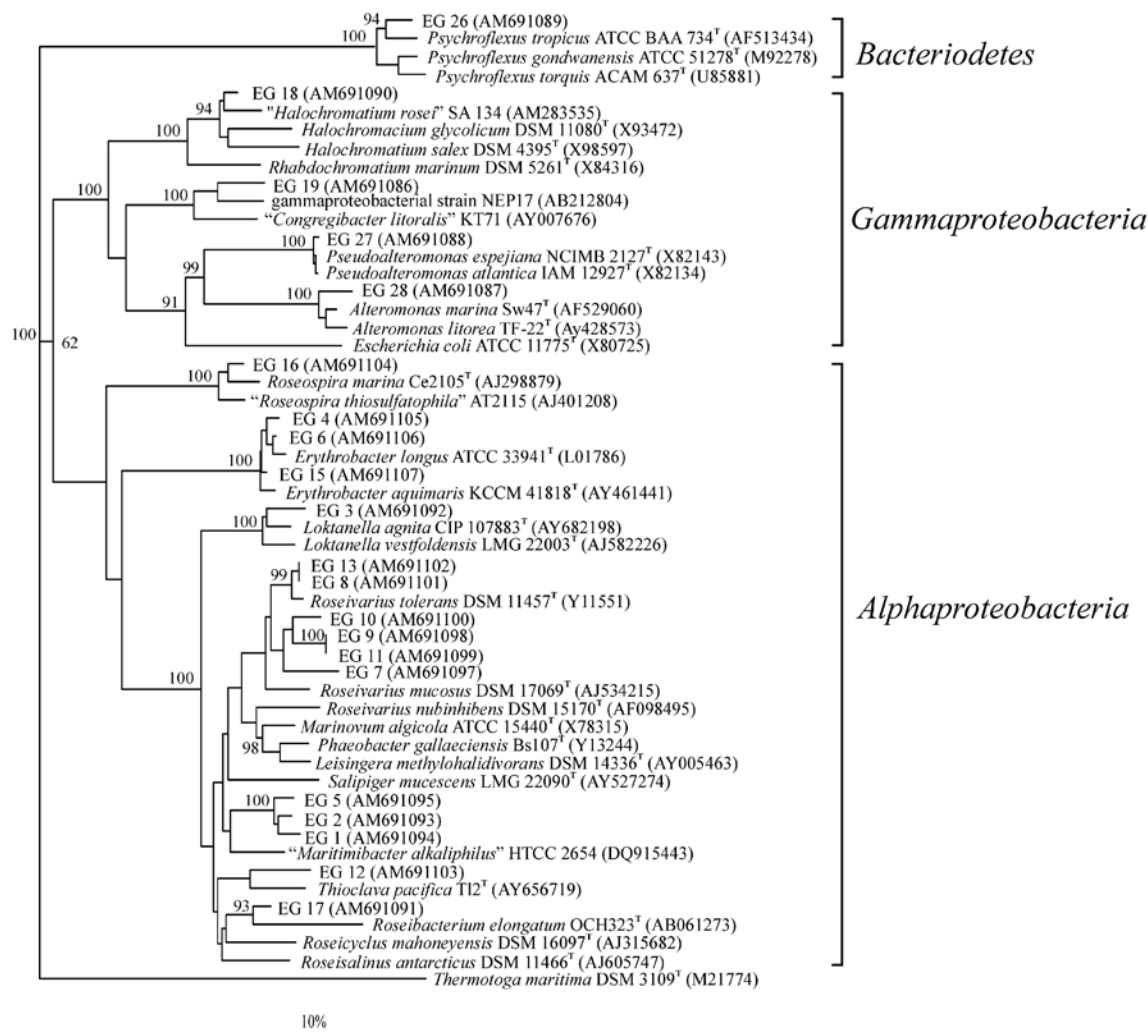
Fig. 2.2. Neighbor-joining phylogenetic tree showing relatedness of 21 EGC isolates based on 16S rDNA sequences more than 1400 nucleotides long. Accession numbers follow strain names. Bootstrap values are indicated at branch points. Quotation marks around Latin names indicate species that have not yet been validated.

relatives of psychrotolerant and halophilic Antarctic and marine chemotrophs such as *Psychroflexus gondwanensis* (EG26, 97.7% sequence similarity) (Bowman *et al.* 1998) and *Pseudoalteromonas carrageenovora* (EG27, 99.7% sequence similarity) (Gauthier *et al.* 1995), the latter being an exclusively marine genus. Delivery may have been mediated by the transpolar migratory arctic tern (*Sterna paradisaea*), a seasonal resident of Manitoba waters. Obligately anaerobic phototrophs (e.g. EG16, EG18) were phylogenetically most similar to halophilic *Roseospira marina* (98.1%) and *Halochromatium glycolicum* (95.6%), the former also described from a hypersaline spring system (Guyoneaud *et al.* 2002). A facultatively anaerobic PNSB, EG17, was related to halotolerant AAP taxa such as *Roseisalinus antarcticus* (95.4%) (Labrenz *et al.* 2005).

AAP exhibited marked novelty, with 9 of the 15 sequenced strains bearing 96% or less 16S rDNA sequence similarity to taxonomically characterized species (Fig. 2.2). Only a few strains (e.g. *Erythrobacter* relatives, EG4, EG6, EG15; >98.5% sequence similarity to *Erythrobacter* species and to each other) formed tight phylogenetic clusters. The considerable heterogeneity displayed by the *Roseivarius tolerans*-related cluster (EG7–EG11, EG13; 95.5–98.6% sequence similarity with *R. tolerans*) was probably due to either numerous delivery events from multiple sources or extensive local evolutionary divergence. Eleven of 15 AAP were members of the α-3 proteobacterial *Roseobacter* clade, many of which hail from hypersaline environments (Yurkov and Csotonyi 2003). For several AAP (EG1, EG2, EG5 92.7-95.2% similar to *Maritimibacter alkaliphilus*; EG3, 96.7% similar to *Loktanella vestfoldensis*; EG12, 94.5% similar to *Thioclava pacifica*), the closest taxonomically described relatives were non-phototrophs (Lee *et al.*

2007; Sorokin *et al.* 2005; Van Trappen *et al.* 2004), reflecting the deep phylogenetic interspersion of AAP with non-phototrophs among the *Proteobacteria*, and probably granting these strains novel genus status on the basis of a significant metabolic distinction and phylogenetic distance.

Of greatest phylogenetic significance, strain EG19 was a member of a recently uncultivable clade (NOR5/OM60), and is only the second reported gammaproteobacterial AAP (92.9% 16S rDNA sequence similarity to *Congregibacter litoralis*). Until the description of gammaproteobacterial *C. litoralis* (Fuchs *et al.* 2007), AAP encompassed 29 alphaproteobacterial genera and one betaproteobacterial genus (Yurkov and Csotonyi 2003, 2009). *Congregibacter* represents a marine clade previously known only from Monterey Bay BAC clones (Fuchs *et al.* 2007). Cultivating such previously unaccessible groups is tremendously beneficial, as these organisms can thereafter be investigated in greater detail than could their environmental gene sequences alone.

2.4.6. Brief phenotypic characterization of isolates

Reflecting their phylogenetic variety, AAP from EGC demonstrated wide phenotypic diversity. Although no novel morphologies were observed, cell shape spanned the gamut from coccoid or ovoid cells (EG2, EG5) (Fig. 2.3a) to short rods (EG3, EG10, EG15) (Fig. 2.3b, c), curved rods (EG19) (Fig. 2.3d) or very long rods (EG6) (Fig. 2.3e), some with tapered ends (EG12) (Fig. 2.3f).
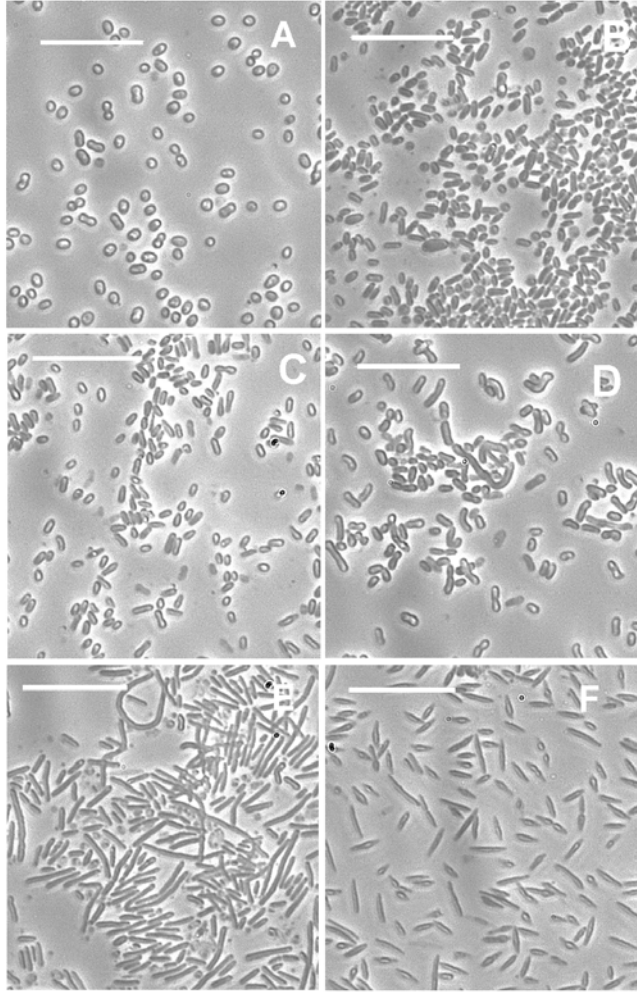
Fig. 2.3. Phase contrast microscopy of strains. **(a)** EG2, pale pink *Maritimibacter* relative. **(b)** EG3, beige *Loktanella* relative. **(c)** EG10, bright pink *Roseivarius* relative. **(d)** EG19, pinkish-orange gammaproteobacterium. **(e)** EG6, reddish-orange *Erythrobacter* relative. **(f)** EG12, pale pink *Thioclava* relative. Scale bars: 10 μm.

2.4.6.1. Spectrophotometric characteristics

In vivo spectrophotometry revealed that most AAP possessed a photosynthetic apparatus whose core elements were the typical *Erythrobacter* type (EG4, EG6, EG15): a single light harvesting (LH) complex (λ_{max} ~800, 870 nm) and reaction center (RC) (λ_{max} ~800 nm) (Yurkov 2006). Several phylogenetically novel AAP, such as the *Maritimibacter*-related cluster (EG1, EG2, EG5) and gammaproteobacterial EG19 (Fig. 2.4a) also shared this property. These organisms were principally distinguished by their carotenoid profiles (Table 2.3).

However, three phylogenetic/spectral classes were particularly intriguing either in BChl spectral properties or in levels and conditions of expression of their photosynthetic apparatus. First, although *Thioclava*-related EG12 incorporated BChl into an *Erythrobacter*-like photosynthetic apparatus (λ_{max} 803 and 869 nm), the extracted BChl (acetone/methanol 7:2, *v/v*) was blue-shifted 18 nm (λ_{max} 752 nm) from the typical 770 nm λ_{max} of Mg-ligated BChl *a* (Fig. 2.4b). This component may be bacteriopheophytin resulting from an easily metal-stripped form of BChl *a*, or it may be or Zn-chelated BChl *a*, thus far found only in acidophilic AAP, *Acidiphillium* and *Acidisphaera* (Hiraishi and Shimada 2001). Detailed BChl structural analysis will be required to discriminate between competing explanations.

Second, the *Loktanella*-related EG3 produced no BChl when cultured at 28$^{\mathrm{o}}$C in the dark for 12 days, (conditions under which most AAP produce BChl), but a near-infrared BChl peak (λ_{max} 862 nm) was detectable after 9 months at 7$^{\mathrm{o}}$C in the dark (Fig. 2.4c). Thus it resembled several strains of *Roseivarius tolerans*, which sythesized BChl
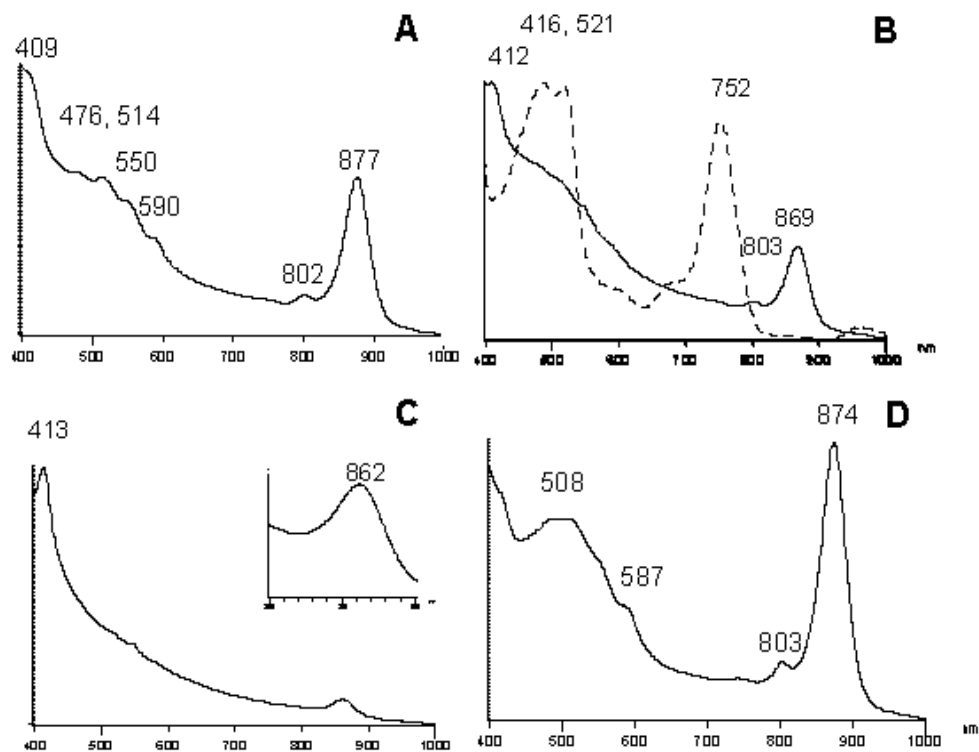
Fig. 2.4. Absorption spectra of phototrophic strains. **(a)** EG19, pinkish orange gammaproteobacterium; *in vivo* spectrum. **(b)** EG12, pale pink *Thioclava* relative; *in vivo* spectrum (solid line) and organic extract in acetone/methanol (7:2 *v/v*) extract (dotted line). **(c)** EG3, pale brown *Loktanella* relative, with inset highlighting BChl peak; *in vivo* spectrum. **(d)** EG9, reddish-purple *Roseivarius* relative; *in vivo* spectrum. Numerals above spectra denote λ_{max} values.

Table 2.3. Phenotypic characteristics of representative AAP isolates.

Strain (& Source)	Color [a]	Cell Shape & Size (μm)	Infrared BChl Absorption Peaks (nm)	Carotenoid and Other Absorption Peaks (nm)	% NaCl Tolerance Full Range & [Optimum]	pH Tolerance Full Range & [Optimum]	Carbon Sources Used for Growth [b]
Erythrobacter-related							
EG4 (Site 1 peripheral mat)	R-O	long rod (1.5-6.3 x 0.5)	803, 867	422, 476	0-14 [4]	6-10 [7-8]	1,2,5,13, 18,20
EG6 (Site 1 peripheral mat)	R-O	long rod (2.0-14.5 x 0.5)	805, 865	417, 465, 482	2-10 [6]	7-10 [7-9]	1,2,5, 18-20
EG15 (Site 5 peripheral mat)	B-R	rod (1.2-4.2 x 0.6)	806, 866	418, 480	2-12 [4]	7-11 [7-9]	2,5,7,10, 18-20
Maritimibacter-related							
EG1 (Site 1 peripheral mat)	P-Bg	ovoid to rod (1-2.2 x 0.7)	801, 867	411, 509, 547	0-22 [12]	7-10 [7-8]	1,5-14, 18-20
EG2 (Site 1 peripheral mat)	pale P	coccoid (0.9-1.2 Ø)	802, 867	416, 481, 514, 549	2-14 [10]	NA	1,3,5-14, 16,19,20
EG5 (Site 1 peripheral mat)	R-Pr	ovoid (1.0-1.7 x 0.8)	803, 867	406, 477, 510, 550	NA	7-11 [7-9]	NA
Loktanella-related							
EG3 (Site 1 peripheral mat)	Bg	rod (1.2-2.0 x 0.7)	862	413, 548	0-12 [4]	NA	2,4,7,8, 10-14, 18-20
Roseivarius-related							
EG7 (Site 2 benthos)	R-Pr	NA	802, 879	409, 481, 504, 548	NA	7-10 [7]	NA
EG8 (Site 2 floating mat)	P-O	rod (1.2-2.0 x 0.6)	801, 870	412, 473, 506, 550	0-22 [2]	7-12 [7-8]	1,2,5-11, 14,16,18-20
EG9 (Site 3 diatom mat)	R-Pr	rod (1.5-2.8 x 0.7)	803, 874	414, 489, 508, 551	NA	7-11 [8]	NA
EG10 (Site 3 diatom mat)	bright P	rod (1.1-2.2 x 0.7)	801, 870	415, 475, 509, 549	0-22 [4]	7-12 [7-9]	1,2,5,7-10, 15,16,18-20

Table 2.3. (continued)

EG11 (Site 3 diatom mat)	R-Pr	rod (1.5-3.0 x 0.5)	801, 874	414, 479, 509, 548	2-26 [18]	7-11 [7-8]	1,2,5,7-11, 16,19,20
EG13 (Site 4 benthic mat)	P-O	rod (1.7-2.8 x 0.5)	801, 870	411, 479, 511, 549, 642	0-22 [14]	7-11 [7-8]	1,5-11,16, 18-20
Thioclava-related							
EG12 (Site 4 benthic mat)	pale P	tapered rod (2-3.8 x 0.5)	803, 869	412, 476, 512, 548	0-18 [6]	7-11 [7-8]	18-20
Gammaproteobacteria							
EG19 (Site 3 diatom mat)	P-O	rod to spirilloid (1.5-3 x 0.7)	802, 877	409, 476, 514, 550	NA	7-12 [7]	NA

[a] R-O, reddish-orange; B-R, brownish-red; P-Bg, pinkish-beige; P, pink; Bg, beige; R-Pr, reddish-purple; P-O, pinkish-orange; [b] 1, acetate; 2, butyrate; 3, citrate; 4, formate; 5, glutamate; 6, glycolate; 7, lactate; 8, malate; 9, propionate; 10, pyruvate; 11, succinate; 12, fructose; 13, glucose; 14, sucrose; 15, ethanol; 16, glycerol; 17, methanol; 18, bactopeptone; 19, casamino acids; 20, yeast extract; Ø, diameter; NA, not applicable

(λ_{max} 877 nm) only after long term cold storage (Labrenz *et al.* 1999), implicating environmental cues in the induction of photosynthesis genes. A satisfactory understanding of the regulation of photosynthetic expression in AAP is far from complete, and because research with *Dinoroseobacter shibae*, *Hoeflea phototrophica* and *Labrenzia alexandrii* suggests that several factors (e.g. illumination regime and nutritional status) can interact in complex and species specific ways (Biebl and Wagner-Döbler 2006), investigation of new species with fastidious BChl expression, such as EG3, will help to complete the picture.

Third, in surprising contrast to the pale beige EG3, the intensely pigmented reddish-purple *Roseivarius*-related cluster (EG7-EG11, EG13) produced uncharacteristically prodigious BChl. *In vivo* peak height ratios of BChl:carotenoids (1.33:1) (Fig. 2.4d) rivaled anaerobic values in PSB (EG18, 1.51:1) or PNSB (EG16, 1.59:1). Most AAP exhibit values around 1:8 to 1:10 (Yurkov and Csotonyi 2003); comparable AAP ratios occur only in Australian strains OCh 245 (1.40:1) and OCh 303 (1.31:1) (Shiba *et al.* 1991). Although unusual PNSB such as *R. centenaria* exist in which O_2 does not inhibit BChl production (Stadtwald-Demchick *et al.* 1990), our strains could not grow anoxically on either Medium B or modified CENMED medium, designed especially for *R. centenaria*. Unusually plentiful BChl suggests a more extensive reliance on the photosynthetic apparatus than in most AAP. Even more unprecedented for AAP, in which light usually completely inhibits BChl synthesis (Yurkov 2006), was the relatively uninhibited BChl synthesis in illuminated EG13 cultures (46% of the maximal dark value was produced under 500 lux illumination, the highest value for any AAP yet described). Alleviation of light-inhibited BChl synthesis would minimize diurnal BChl dilution in

rapidly dividing eutrophic populations. Species of AAP producing copious BChl are invaluable to studies of the intriguing biophysics of light-mediated energy transduction in AAP.

2.4.6.2. Salinity and pH tolerance

Most AAP preferred neutral to weakly basic pH for growth, similar to their native habitat pH (Table 2.1), but EG8, EG10 and EG19 tolerated pH 12 (Table 2.3), making them well adapted to the elevated pH generated diurnally in microbial mats by photosynthesis. EGC also yielded strains with the highest known halotolerance in AAP. All tested isolates tolerated NaCl concentrations of at least 6%. Some exhibited optimal growth at the highest salinity (14 or 18% NaCl; EG13, EG11) yet reported for AAP (Table 2.3). Others tolerated the greatest salinity range (2–26%, EG11) and the highest upper salinity limit for growth (22 or 26% NaCl; EG1, EG8, EG11). The previous maximum NaCl concentration endured was 20% by *R. halodurans, R. halotolerans* and *S. guttiformis* (reviewed by Yurkov and Csotonyi 2003). Most saline environments screened for AAP are salt, soda or meromictic lakes, but the few investigated marine tidal salt flats have yielded considerably halotolerant AAP, such as *Erythrobacter litoralis* (0.5 to 9.6% NaCl) and *Roseibium denhamense* (0 to 10% NaCl) (reviewed by Yurkov and Csotonyi 2003). However, presence of a diverse halotolerant microbial community 600 km from the ocean (Hudson Bay) is noteworthy. McKillop *et al.* (1992) speculated on waterfowl-mediated delivery of marine invertebrates to the springs, which may also explain rapid colonization of the EGC region by marine foraminifera from the Gulf of Mexico during the Holocene Hypsithermal, about 5500 years ago (Patterson *et al.* 1997).

2.4.6.3. Organic carbon requirements

As for Mahoney Lake (Yurkova *et al.* 2002), AAP isolates fell into three clusters based on organic carbon utilization profiles (Table 2.3). Phylogenetically diverse cluster A (EG1, EG2, EG3, EG8, EG10, EG11, EG13) grew on most (11 to 14) carbon sources tested. Cluster B (*Erythrobacter* relatives, EG4, EG6 and EG15, with representatives recovered from every site sampled) utilized 6 to 7 substrates. Most interestingly, Cluster C (*Thioclava* relative, EG12) used only complex carbon sources (Bactopeptone, casamino acids and yeast extract) and could not be cultured on defined media, reflecting a likely adaptation to niches providing a reliable source of a single nutrient.

2.5. Conclusions

Aquatic environments that are geographically discontinuous with each other, the ocean or a parent river system resemble ecological island habitats in which endemism is expected to be high (MacArthur and Wilson 1967). The physically extreme conditions of saline environments such as meromictic lakes and the EGC saline springs further isolate them biologically from their surroundings. Surveys of their biodiversity are hence not only invaluable to establishing proper environmental protection policies for rare habitats, but especially constructive to our understanding of the phylogenetic extent, evolution, and physiology of groups such as the AAP, whose great relative global abundance, and thus biogeochemical influence, are only now being recognized (Yurkov and Csotonyi 2009).

2.6. Acknowledgements

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Chapter 3.

Chromocurvus halotolerans, gen, nov., sp. nov., a gammaproteobacterial obligately aerobic anoxygenic phototroph, isolated from a Canadian hypersaline spring.

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Peter Schumann, and Vladimir Yurkov

The first author was the major contributor to research presented.

3.1. Abstract

A strain EG19^T of aerobic bacteria able to form pleomorphic cells was isolated from a brine spring runoff stream in the west central region of the Province of Manitoba, Canada. The pale pinkish purple strain contained bacteriochlorophyll *a* incorporated into light harvesting I and reaction center complexes. Its inability to grow under anaerobic illuminated conditions prompted designation as a member of the functional group known as aerobic anoxygenic phototrophic bacteria. Phylogenetic analysis of the 16S rRNA gene sequence revealed that it belonged to the *Gammaproteobacteria*, forming a distinct branch of phototrophs distantly related to most described aerobic anoxygenic phototrophs, quite marginally related (95.6%) both to the only other described gammaproteobacterial aerobic phototroph, *Congregibacter litoralis* and also to non-phototrophs in the genus *Haliea* (95.1–96.1%). Physiological tests demonstrated tolerance profiles to salinity (0–18% NaCl), pH (7–12) and temperature (7–40°C) consistent with survival in a shallow hypersaline stream on the exposed, vegetation-depleted salt playa of its native East German Creek. Phylogenetic data and phenotypic properties such as pigment composition, morphology and physiology support the proposal of the novel genus and species *Chromocurvus halotolerans* gen. nov., sp. nov., with EG19^T (= DSM 23344^T, = VKM B-2659^T) as the type strain.

3.2. Introduction

East German Creek (EGC) is an unusual inland pH-neutral hypersaline spring system located in the west center of the province of Manitoba, Canada. Although separated from the ocean by over 600 km, this extreme environment possesses source water with twice marine salinity (total dissolved solids 56.7–67.3‰) (Csotonyi et al. 2008) and salt-encrusted playas where salinity reaches saturation (McKillop et al. 1992; Csotonyi et al. 2008). Sulfide from a subterranean community of sulfate reducers supports purple sulfur and purple nonsulfur bacteria near the surface of microbial mats. However, high flow rate of springs (up to 4800 l/h; McKillop et al. 1992) and prodigious growth of diatoms and the marine green algae *Percursaria percursa* (Londry et al. 2005; Csotonyi et al. 2008) oxygenates surface microbial communities, where an abundant and diverse community of aerobic anoxygenic phototrophs (AAP) was recently enumerated (Csotonyi et al. 2008).

AAP typically contain over ten-fold less light harvesting pigment bacteriochlorophyll (BChl) than anaerobic anoxygenic phototrophs, relying mainly on heterotrophic carbon utilization instead of photosynthesis. They are incapable of autotrophy (Yurkov and Csotonyi 2009). AAP are obligately aerobic, requiring oxygen for their growth, for the incorporation of BChl into functional reaction center (RC) and light harvesting (LH) complexes, and for photosynthetic function (Yurkov and Beatty, 1998; Rathgeber *et al.*, 2004; Yurkov and Csotonyi 2009). BChl synthesis is typically inhibited by light (Yurkov and Csotonyi 2009). AAP also abundantly produce from one to over twenty different carotenoids per species (Fuchs et al. 2007; Yurkov and Beatty 1998), most of which are disengaged from photosynthetic energy transduction (Noguchi et al. 1992; Yurkov et al.

1993). Despite these features that distinguish them from other anoxygenic phototrophs, AAP were not recognized as a group until the late 1970s (Shiba et al. 1979). Since then, their global prominance is increasingly being realized. The AAP constitute a sizable proportion of microorganisms in several major ecosystems worldwide: up to 18% of the microbiological community in the world ocean (reviewed by Yurkov and Csotonyi 2009), and an even larger percentage (up to 36%) of cultivable cells in extreme environments (Csotonyi et al. 2008, Yurkov and Csotonyi 2003).

Fifty-five of the 57 currently described species of AAP are related most closely to alphaproteobacterial purple nonsulfur bacteria (Boldareva et al. 2009; Yurkov and Csotonyi 2009). Only one species, *Roseateles depolymerans*, is known from the *Betaproteobacteria* (Suyama et al. 1999). Prior to the recent description of the first gammaproteobacterium, *Congregibacter litoralis* (Fuchs et al. 2007), the presence of gammaproteobacterial AAP had only been hypothesized from bacterial chromosome library sequences containing unusual photosynthesis genes from Monterey Bay seawater (Béjà et al. 2002). However, the NOR5/OM60 clade, to which *C. litoralis* belongs, is widely distributed across earth's saline environments (Yan et al. 2009), implying that gammaproteobacterial AAP are globally important.

The aim of the present study was to describe a novel species, the second known gammaproteobacterial AAP, isolated from a hypersaline EGC stream (Csotonyi et al., 2008). Our study reinforces the idea that the extent of diversity of gammaproteobacterial AAP has only been scratched at the surface by microbiologists.

3.3. Materials and methods

3.3.1. Isolation and cultivation

A microbial mat rich in diatoms was sampled from an outflow stream about 100 m from a spring at EGC in May 2002, as previously described (Csotonyi et al. 2008). Strain EG19^T was isolated from the agar surface of an illuminated agar deep culture on Medium B (Csotonyi et al. 2008), designed for purple nonsulfur bacteria. It was subsequently cultivated on Medium A (Csotonyi et al. 2008), a rich organic variant for AAP from EGC. This medium was used for cultivation in continual darkness at 28°C, unless otherwise noted.

3.3.2. Photosynthetic pigment analysis

Presence of BChl *a* and carotenoids was assayed spectrophotometrically (Hitachi U-2010) in 5-day-old aerobic cultures on Medium A. Absorbance properties of pigments incorporated into the RC and LH complexes were determined *in vivo* as described in Yurkov and van Gemerden (1993). BChl content was determined from acetone/methanol (7:2) extracted pigments, calculated from A_{770} and biomass (Yurkov and van Gemerden 1993). Anaerobic photosynthetic growth was assayed in the presence of alternative electron donors as previously described (Csotonyi et al., 2008).

3.3.3. Phenotypic characterization

Cellular morphology was determined by phase contrast microscopy (Zeiss Axioskop 2) in late exponential-phase agar cultures grown aerobically on Medium A. Tolerance to pH was determined as previously described (Csotonyi et al. 2008). Tolerance to NaCl was determined by OD_{660} measurements after 1, 2 and 5 days of incubation at $28^{o}C$ in rich organic medium for AAP (Yurkov 2006) to which NaCl was added at concentrations ranging from 0 to 30%, in increments of 2%. The temperature range for growth was examined by scoring growth on agarized Medium A at temperatures of 2, 7, 12, 28, 37, 40 and $45^{o}C$. Organic carbon utilization was tested using basal carbon-free Medium A (Csotonyi et al. 2008), for the following components added singly at a concentration of 1 g/l: acetate, bactopeptone, butyrate, casamino acids, citrate, ethanol, formate, fructose, glucose, glutamate, glycerol, glycolate, lactate, malate, methanol, propionate, pyruvate, succinate, sucrose and yeast extract. Autotrophy was tested by measuring growth in three serial transfers of aerobically incubated liquid basal Medium A amended with 1.5 g/l $NaHCO_3$. Physiological tests for antibiotic resistance, fermentation, presence of lipase, gelatinase, catalase and oxidase were determined as previously described (Yurkov et al. 1994; Csotonyi et al. 2008). Requirement for vitamins was tested on three successive subcultures in tubes of liquid basal Medium A amended with the carbon sources glutamate, pyruvate, acetate, lactate and malate (1 g/l each), and with vitamin solutions (Yurkov 2006) from which one of the following was removed for each treatment: biotin, B_{12}, nicotinic acid or thiamine.

3.3.4. 16S rRNA gene sequence analysis and DNA G+C content determination

Genomic DNA was extracted for phylogenetic analysis, and the nearly complete 16S rRNA gene sequence (greater than 1400 nucleotides long) was analyzed. PCR-amplification, sequencing, electrophoresis, sequence alignment with published sequences, similarity value determination, dendrogram construction and bootstrap value determination were carried out as described by Csotonyi et al. (2008). The sequence accession number AM691086 was obtained for EG19^T. DNA G+C content was determined by using HPLC of nucleotides obtained according to Mesbah et al. (1989).

3.4. Results and discussion

3.4.1. Culture properties and morphology

Strain EG19^T formed small, ~ 1-2 mm-diameter pale pinkish-purple colonies tinged slightly with orange on the surface of agar media, and bore an adhesive consistency. In aerobic liquid media, cultures were more markedly orange-pink, achieving this color in 24 hours. In both liquid and agar cultures, incubated with yeast extract (1 g/l), a brownish orange extracellular component was released that diffused through the medium, becoming more pronounced after several days of growth. This component was responsible for the orange tinge of cultures, remaining in the supernatant following centrifugation.

EG19^T was pleomorphic, ranging from short rod-shaped to bent and irregularly shaped cells, 1.5-3.0 x 0.7 µm in size (Fig. 3.1). The phylogenetically closest and taxonomically described phototrophic relative of EG19, *Congregibacter litoralis* KT71, is a coccoid to rod-shaped AAP (Fuchs et al. 2007). Nonlinear cells are rarely reported in AAP. Recently, Rathgeber et al. (2005) described the sharply curved vibrioid cells of the α-3 proteobacterial AAP, *Roseicyclus mahoneyensis*. Sieracki et al. (2006) observed uncultivated spirilloid cells in the Sargasso Sea that they interpreted as obligately aerobic phototrophs, but these organisms were not cultivated, and therefore their identity as AAP could not be confirmed. Motility was observed in EG19^T, a characteristic that it shared with *C. litoralis*. Unlike EG19^T, the closest non-photosynthetic relatives, *Haliea rubra* and *Haliea salexigens*, produce rod-shaped cells (Urios et al. 2008; 2009). Of them, only *H. salexigens* is motile.

3.4.2. Photosynthetic pigment analysis

Absorbance properties of pigments incorporated into the RC and LH complexes are shown in Fig. 3.2. The *in vivo* spectrum exhibited near-IR peaks at 802 and 877 nm (Fig. 3.2a), indicative of BChl *a* incorporated into RC and a single LH complex. The LH complex was remarkably red-shifted from the typical B870 LH I (Yurkov and Csotonyi 2009), absorbing more like the 876 nm LH I of *C. litoralis* (Spring et al. 2009). Under strictly heterotrophic and eutrophic conditions, when *C. litoralis* never synthesized BChl, EG19^T produced up to 1.11 nmol BChl / mg dry weight of cells, or about one third of the BChl *a* synthesized by *C. litoralis* under optimally photoheterotrophic (oligotrophic)
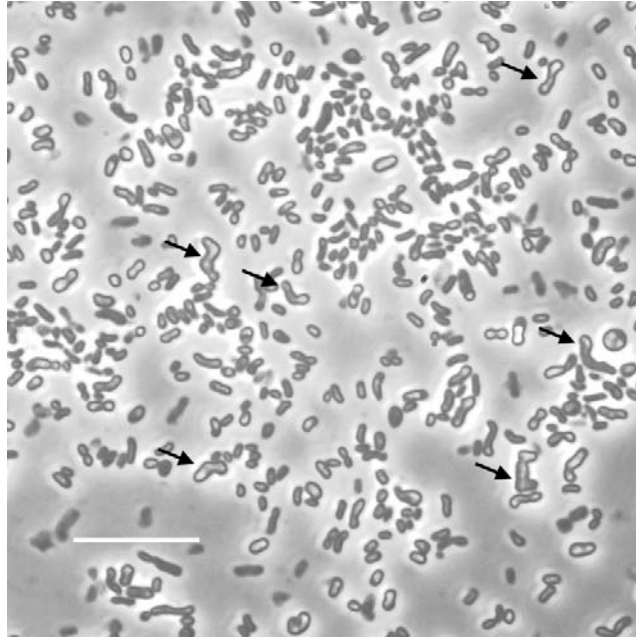
Fig. 3.1. Phase contrast microscopy of pleomorphic strain EG19^T. Arrows indicate examples of bent and irregularly shaped cells. Scale bar: 10 μm.
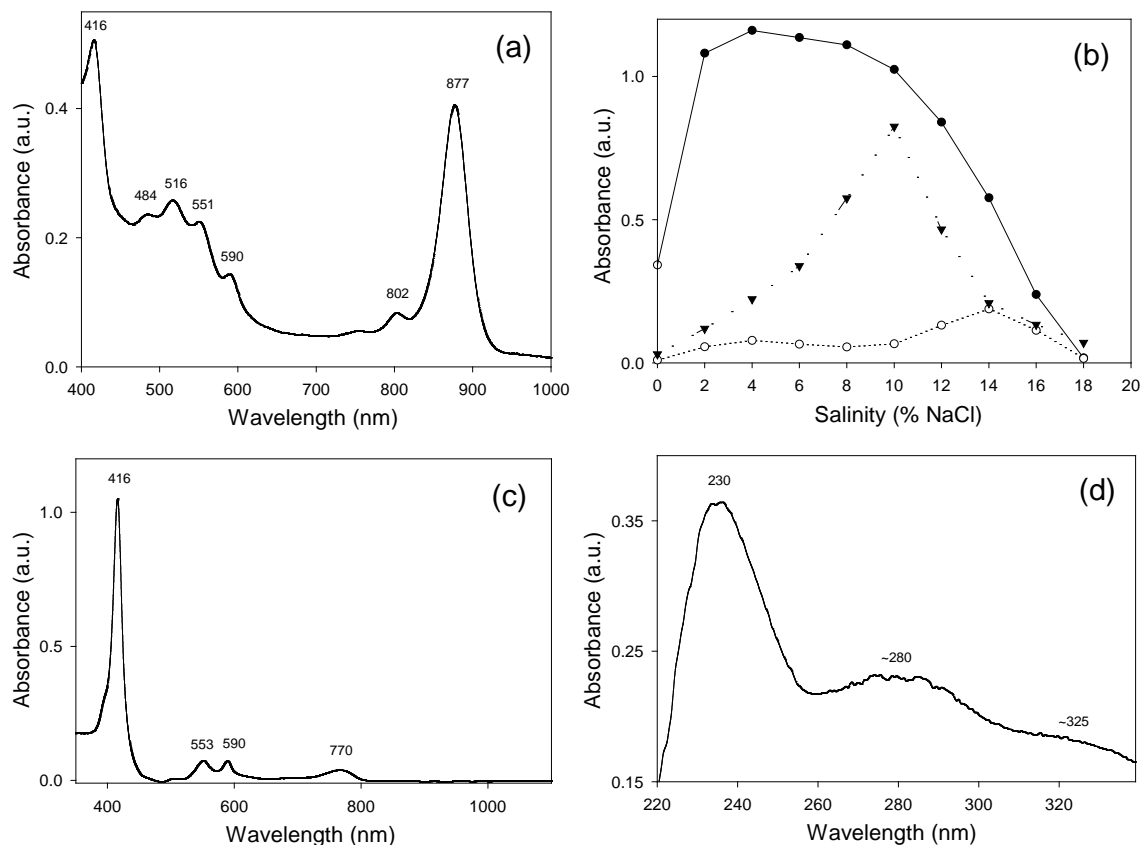
Fig. 3.2. Photosynthetic apparatus and pigmentation in EG19[T]. **(a)** *In vivo* spectrum. **(b)** Salinity dependence of biomass yield (OD_{660}) (–●–), BChl production (–○–) and accumulation of a pigment absorbing maximally at 416 nm (–▼–), both latter components standardized to biomass (OD_{660}). **(c)** Difference spectrum between extracts from cultures grown at 2 and 10% NaCl, exhibiting absorption peaks (416, 553, 590 nm) suggestive of the BChl precursor, Mg protoporphyrin IX, expressed maximally at 10% NaCl. **(d)** *In vivo* UV spectrum of supernatant from centrifuged liquid culture of EG19[T], showing absorption peaks of brownish diffusible water soluble pigment. Numerals above spectra, λ_{max} values.

conditions (3.02 $\pm$ 0.42 nmol BChl / mg dry weight of cells; Spring et al. 2009). These

values of BChl content fall in a range that is typical for AAP (Yurkov and Csotonyi

2009). *In vivo* values of $A_{877\,nm}/A_{660\,nm}$, an estimate of biomass-specific LH I abundance,

ranged from 0.54 to 0.74 for EG19, compared with values of 0.94 to 1.05 for *C. litoralis*

(Spring et al. 2009). Presence of photosynthetic pigments distinguishes EG19[T] from its

nearest phylogenetic neighbours, cream-colored *H. salexigens* and red *H. rubra*, from

which BChl was not reported (Urios et al. 2008; 2009).

Strain EG19[T] did not use BChl for anaerobic photosynthesis with either acetate, malate,

succinate, thiosulfate, or sulfide as electron donor. Growth did not occur anaerobically in

either illuminated or dark conditions, and light was not required for aerobic growth.

These observations led us to designate EG19[T] as an AAP (Yurkov and Csotonyi 2009).

Carotenoids absorbed maximally *in vivo* at 484, 516 and 551 nm (Fig. 3.2a), and at 467,

495 and 529 nm in extract (Table 3.1). This absorption profile strongly suggested an

identity of spirilloxanthin, the dominant carotenoid pigment in many anaerobic

gammaproteobacterial phototrophs (Takaichi 2004). In this regard, EG19[T] resembled *C.

litoralis*, which also contained only the carotenoid spirilloxanthin (Spring et al. 2009).

Pigmentation in EG19[T] was highly dependent upon salinity of the culture medium, with

the cellular content of BChl ranging from 0.07 to 1.11 nmol BChl / mg dry weight of

cells (Fig. 3.2b). Although growth rate was optimal at 8% NaCl and yield was highest at

4%, BChl production was maximal at 14% NaCl, near the upper limit for growth (18%

Table 3.1. Distinguishing phenotypic traits of *Chromocurvus halotolerans,* EG19[T] compared to *Congregibacter litoralis*, KT71[T]; *Haliea rubra*, CM41_15a[T]; and *Haliea salexigens*, 3X/A02/235[T].

Characteristic	EG19[T]	KT71[T]	CM41_15a[T]	3X/A02/235[T]
Isolation source	Central Canadian brine spring runoff stream	North Sea (8 m depth)	Mediterranean Sea (3 m depth)	Mediterranean Sea (surface)
Morphology	Pleomorphic (short rods to bent or irregularly shaped)	Pleomorphic (cocci to rods)	Rods	Rods
Motility	+	+	-	+
Growth characteristics				
NaCl range (optimum) (%)	0-18 (4)	1-7 (2)	0.7-4.2 (3.5)	0.7-7 (4.2)
pH range (optimum)	7-12 (7)	6.5-9 (7.5-8)	5-9 (8)	5-9 (8)
Temp. range (optimum) (^{o}C)	7-40 (37)	9-33 (28)	15-44 (30)	10-37 (25-30)
Anaerobic growth	-	-	-	-
Autotrophic growth	-	-	n.a.	n.a.
Enzymes				
Catalase	+	(+)	+	+
Oxidase	-	+	+	+
Pigmentation				
BChl absorption (*in vivo*) (nm)	802, 877	802, 876	n.a.	n.a.
Carotenoid absorption, acetone/methanol, (*in vivo* in parentheses) (nm)	467, 495, 529 (484, 516, 551)	465, 495, 529	n.a.	n.a.
Diffusible compound	+	-	-	-
Utilization of:				
Acetate	+	(+)	-	-
Butyrate	-	(+)	n.a.	n.a.
Citrate	-	-	+	-
Formate	-	-	n.a.	n.a.
Glutamate	(+)	+	-	(+)
Glycolate	-	-	n.a.	n.a.
Lactate	-	-	-	-
Malate	(+)	+	n.a.	n.a.
Propionate	-	(+)	-	-
Pyruvate	+	+	-	+
Succinate	(+)	+	-	+
Fructose	-	-	(+)	-
Glucose	-	-	+	-
Sucrose	-	+	-	-
Ethanol	-	-	n.a.	n.a.
Glycerol	-	+	-	+
Methanol	-	-	n.a.	n.a.
Bactopeptone	+	n.a.	n.a.	n.a.
Casamino acids	+	n.a.	n.a.	n.a.
Yeast extract	+	+	n.a.	n.a.

Hydrolysis of:				
Starch	-	-	n.a.	n.a.
Gelatin	-	-	n.a.	n.a.
Tween 60	-	n.a.	n.a.	n.a.
Antibiotic sensitivity				
Ampicillin	-	n.a.	n.a.	n.a.
Chloramphenicol	+	+	n.a.	n.a.
Imipenem	-	+	n.a.	n.a.
Kanamycin	(+)	n.a.	n.a.	n.a.
Nalidixic acid	+	n.a.	n.a.	n.a.
Penicillin	+	n.a.	n.a.	n.a.
Polymyxin B	+	+	n.a.	n.a.
Streptomycin	-	n.a.	n.a.	n.a.
Vitamin Requirement				
B_{12}	+	+	n.a.	n.a.
Biotin	+	+	n.a.	n.a.
Nicotinic acid	-	-	n.a.	n.a.
Thiamine	+	+	n.a.	n.a.

Data for *C. litoralis*, *H. rubra* and *H. salexigens* acquired from Spring *et al.* (2009), Urios *et al.* (2009) and Urios *et al.* (2008), respectively; -, negative; (+), weak positive; +, positive; n.a., not available.

NaCl) (Fig. 3.2b). This trend reflected the frequently demonstrated phenomenon that suboptimal growth conditions stimulate BChl synthesis in AAP (Yurkov and Csotonyi 2009). However, in all published species of AAP in which the effect of salinity on BChl production was investigated (*Thalassobacter stenotrophicus*, *Hoeflea phototrophica* and *Citromicrobium bathyomarinum*), deviation to lower rather than higher salinity enhances BChl production (Yurkov and Csotonyi 2009).

An additional pigment was produced maximally at 10% NaCl (Fig. 3.2c). The difference spectrum between cultures at 10% and 2% NaCl indicated absorption maxima of 416, 553 and 590 nm. These suggest an identity of Mg-protoporphyrin IX or its monomethyl ester, two successive intermediates in the biosynthesis pathway of BChl (Ouchane et al. 2004). Although some cytochromes also absorb maximally at comparable wavelengths, the component possessed by EG19[T] is unlikely to be a cytochrome because it was successfully extracted by acetone/methanol (7:2), confirming its pigment nature, whereas cytochromes would be denatured and precipitated by such a solvent mixture. Furthermore, the absorption spectrum of the unknown component matched well the absorption maxima of a Mg-protoporphyrin standard (Frontier Scientific) at 417, 551 and 593 nm. Maximal accumulation of this component around the salinity where BChl production is near minimum (8% NaCl) implies that at optimal conditions for heterotrophic growth, the expression of the photosynthetic apparatus may be down-regulated via partial interruption of the synthesis of the primary light harvesting pigment, BChl. Perhaps substrates or cofactors required for this step are competitively co-opted by other biochemical pathways under heterotrophically favourable conditions. No similar

pigment was reported in *C. litoralis*, *H. rubra* or *H. salexigens*, making its conditional presence in EG19^T a potentially useful taxonomical marker.

When cultured with complex carbon sources such as yeast extract or casamino acids, EG19^T excreted a brownish-orange pigmented hydrophilic component that diffused extensively into the surrounding agar or liquid medium. Spectrophotometry of the supernatant of liquid cultures revealed UV absorption maxima at 230 and 280 nm, with a shoulder at 325 nm (Fig. 3.2d). This absorption spectrum distinguished it from the unidentified diffusible water soluble brown pigment produced by members of the non-phototrophic genus *Ketogulonicigenium*, which have a single absorption peak at 310 nm (Urbance et al. 2001). Although the identity of the diffusible pigment of EG19^T is currently unknown, its absorption spectrum shares characteristics with some siderophores, such as 'lotibactin' produced by *Mesorhizobium loti* (Morton et al. 2007), which are iron-binding molecules excreted by some bacteria into their surroundings to sequester Fe under iron-limiting conditions. However the abundance of iron at EGC (Fe oxyhydroxides confer a bright orange color to the entire salt pan) imply that siderophores are not required by resident microorganisms to acquire sufficient quantities of the element. This observation challenges the identity of the component in question as a siderophore. Whatever its function, the copious production of this compound under organic rich conditions may serve as a useful taxonomical marker for the species, as no such production was reported for *C. litoralis*, *H. rubra* or *H. salexigens* (Fuchs et al. 2007; Spring et al. 2009; Urios et al. 2008; 2009)

3.4.3. Phenotypic characterization

A variety of physiological characteristics compared to the most closely related gammaproteobacterial phototroph and the non-phototrophs *H. rubra* and *H. salexigens* are presented in Table 3.1. EG19[T] exhibited very wide salt tolerance, growing with 0 to 18% NaCl and demonstrating most rapid initial growth at 4% (Fig. 3.2b). Near maximal biomass was produced between 0 and 16% NaCl. This trend implies that EG19[T] is well adapted to highly fluctuating salt concentration in its native outflow stream habitat, where meteoric water can periodically dilute the source water (56.7-66.3‰ total dissolved solids; Csotonyi et al. 2008), but desiccation can also raise its salinity to saturation. Adaptation to this widely fluctuating hypersaline environment explains the much greater salinity tolerance limits of EG19[T] compared with the marine *C. litoralis*, *H. rubra* and *H. salexigens* (the widest range 0.7 to 7%) (Table 3.1).

Optimal pH for growth was 7.0, reflecting the pH 7.2 measured in its source stream (Csotonyi et al. 2008). However, EG19[T] also tolerated a wide range of pH, between 7.0 and 12.0, the latter being the highest value that was tested. Oxygenic phototrophs, which constitute the majority of the biomass of EGC mats, can cause large diurnal fluctuations in pH as a result of photosynthesis. EG19[T] was capable of surviving such swings in alkalinity. The near neutral pH of the ocean is much more stable, which is why *C. litoralis*, *H. rubra* and *H. salexigens*, by comparison, tolerate a narrower range in pH (the widest range 5 to 9) (Table 3.1).

EG19^T demonstrated an impressively wide range of tolerance to temperature, from 7°C to 40°C, showing near optimal biomass yield from 12°C to 37°C, with an optimum at 37°C. The EGC salt playa is nearly devoid of vegetation (McKillop et al. 1992; Csotonyi et al. 2008). Therefore, it is fully exposed to solar radiation in the summer, and lacks a layer of thermal insulation against periods of cold during autumn, winter and spring. The temperature tolerance profile of EG19^T reflected the fact that organisms thriving in this environment must be able to withstand a widely fluctuating thermal regime. By contrast, the ocean's thermal regime is generally quite stable. For this reason, all three nearest relatives had lower optimum temperatures than EG19^T (Table 3.1), and the temperature range for growth of EG19^T was 9°C, 8°C and 10°C wider than those of *C. litoralis*, *H. rubra* and *H. salexigens*, respectively (Table 3.1).

Heterotrophic growth occurred in defined media amended with the organic compounds acetate, glutamate, malate, pyruvate and succinate. Biomass yield was best on the complex carbon sources bactopeptone, casamino acids and yeast extract. No fermentation of glucose, sucrose or fructose was observed. EG19^T could not grow with inorganic carbon alone. This finding further supported its identification as an AAP, none of which possess the capacity for autotrophy (Yurkov and Csotonyi 2009). In this way, EG19^T was similar to *C. litoralis*, the completely sequenced genome of which revealed the absence of key Calvin cycle genes (Fuchs et al. 2009). Heterotrophically, however, EG19^T exhibited a narrower carbon utilization profile than *C. litoralis*, for it could not grow with propionate, sucrose or glycerol as sole carbon sources. Unlike *H. salexigens*, EG19^T could not rely solely on glycerol but grew with acetate. EG19^T differed from *H. rubra* in

that it could not utilize citrate, fructose or glucose, but could metabolize acetate, glutamate, pyruvate and succinate (Table 3.1).

Like *C. litoralis*, strain EG19[T] possessed neither amylase nor gelatinase activity. EG19[T] was incapable of hydrolysing Tween 60, indicating a lack of lipase activity (Table 3.1). Like all of its nearest relatives, EG19[T] produced catalase, but unlike any of them, EG19[T] lacked cytochrome *c* oxidase activity (Table 3.1). Most aerobic bacteria that respire with oxygen as a terminal electron acceptor possess this enzyme, making EG19[T] unusual in this regard and rendering the trait a useful indicator for identification. EG19[T] was resistant to ampicillin (2 μg), imipenem (10 μg) and streptomycin (10 μg), was weakly resistant to kanamycin (30 μg), but was sensitive to chloramphenicol (30 μg), nalidixic acid (30 μg), penicillin G (10 IU) and polymyxin B (300 IU). EG19[T] resembled *C. litoralis*, except in its resistance to imipenem (Table 3.1).

As for *C. litoralis*, biotin, thiamine and vitamin B_{12} were required for growth on defined medium, but EG19[T] could be subcultured repeatedly in the absence of nicotinic acid (Table 3.1). AAP exhibit considerable variety in their requirements for vitamins, some species growing well without any vitamins (e.g. *Roseisalinus antarcticus*; Labrenz et al. 2005), others requiring no vitamins but stimulated by them (e.g. *Roseovarius tolerans*, stimulated by thiamine, nicotinic acid, biotin, and B_{12}; Labrenz et al. 1999), and still others requiring vitamins (e.g. *Porphyrobacter meromictius*, of which all strains require B_{12} and some strains require biotin; Rathgeber et al. 2007).

3.4.4. 16S rRNA gene sequence analysis and DNA G+C content determination

Analysis of the 16S rRNA gene sequence revealed that strain EG19[T] was a member of the *Gammaproteobacteria* (Fig. 3.3). On the basis of the analysis of the algorithm of De Soete (1983), neighbour-joining and maximum-likelihood, stain EG19[T] defined a novel lineage. The phylogenetic distance between EG19[T] and the AAP *C. litoralis* (95.6%) was similar to that which is generally found between well-separated genera. EG19[T] was slightly more closely related to the non-phototrophs *H. rubra* (96.1%), *H. salexigens* (96.0%) and *H. mediterranus* (95.1%), but bore less than 91.4% 16S rRNA gene sequence similarity to any other taxonomically described organism.

The DNA G+C content of EG19[T] was 63.0 mol%, considerably higher than the 57.8 mol% of *C. litoralis* (Table 3.1). Especially in combination with the low phylogenetic overlap between EG19[T] and *C. litoralis*, this datum supports the taxonomic distinction of the two organisms from each other (Wayne et al. 1987). Although DNA G+C values of *H. salexigens* (61.0%) and *H. rubra* (65.0%) agreed more closely, neither was described as phototrophic (Urios et al. 2008; 2009).

In summary, the presence of BChl *a* incorporated into the RC and a LH I complex, the inability to grow phototrophically under anaerobic conditions and the absence of autotrophy prompt us to conclude that strain EG19[T] is indeed a member of the AAP. Phylogenetic analysis demonstrated that EG19[T] represents a distinct phototrophic branch within the *Gammaproteobacteria*, related marginally to the AAP genus *Congregibacter*.
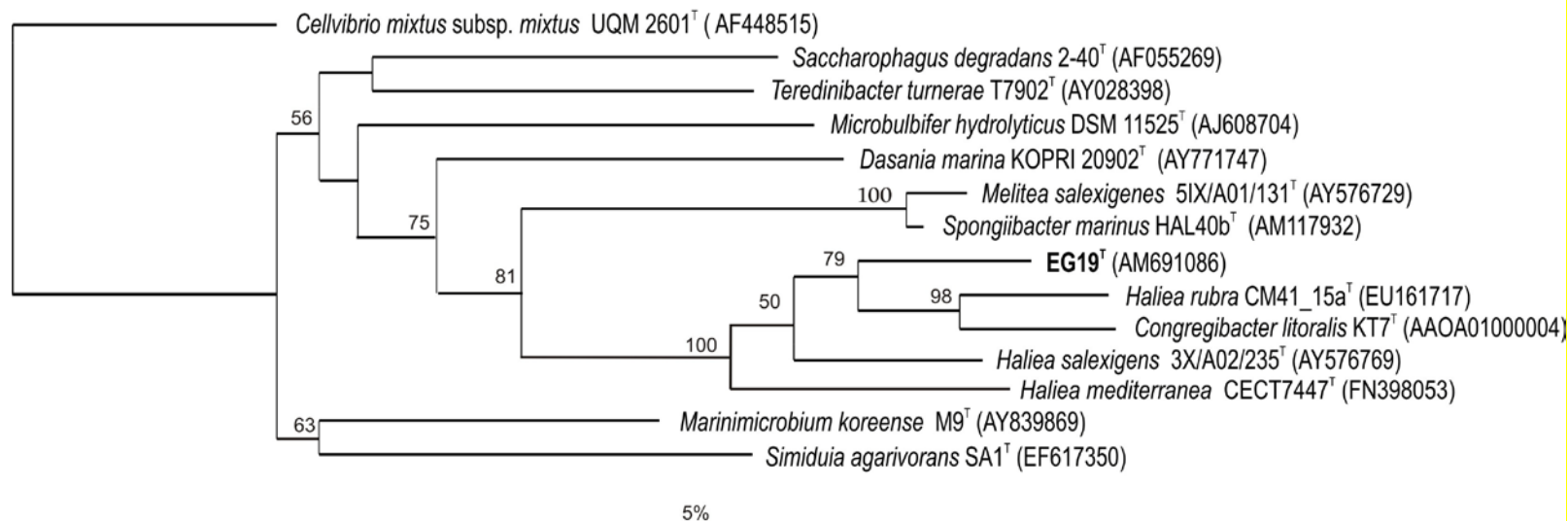
Fig. 3.3. Neighbour-joining dendrogram of 16S rRNA gene sequence relatedness, showing the position of *Chromocurvus halotolerans*, strain EG19[T] and its phylogenetic neighbours, including the gammaproteobacterial AAP *Congregibacter litoralis*. The tree was rooted with 16S rRNA gene sequences of other members of the *Gammaproteobacteria*. Bootstrap confidence values are displayed at branch points. Bar, 5 substitutions per 100 sequence positions.

Although the dendrogram suggests that EG19^T is subtended by the genus *Haliea*, the distance of each species from EG19^T (96.1% for *H. rubra* and 96.0% for *H. salexigens*), the substantially low phylogenetic similarity of the two most closely related *Haliea* species to each other (94.8%), the phototrophic character of EG19^T and numerous phenotypic distinctions argue for erection of a novel genus for EG19^T and the cleaving of *Haliea* into more than one genus rather than incorporation of EG19^T into *Haliea*. The red pigmented *H. rubra* is actually phylogenetically nearer to *C. litoralis* (97.0%) than to either EG19^T (96.1%) or *H. salexigens* (94.8%). Therefore, *H. rubra* may actually belong more appropriately to the genus *Congregibacter* or a novel genus, as its 16S rRNA gene sequence similarity (97.0%) alone would support genus-level distinction. *Haliea mediterranea* was recently described (Lucena et al. 2010), but although it phylogenetically subtends the EG19^T-*Congregibacter-Haliea* cluster, this organism is even more distantly related to EG19^T (95.1%). It is sufficiently different enough from EG19^T (95.1%), *Congregibacter* (94.4%) and the remaining species of *Haliea* (94.7-95.2%) to be described in a new genus. Its lack of photosynthetic pigments further strongly supports genus-level distinction from EG19^T. Additional study to this end should be made and a reclassification, if necessary, should be accomplished. However, such a task is beyond the scope of the current work, which aims simply to describe a novel gammaproteobacterial AAP. Morphological, physiological and biochemical properties allowed us to easily differentiate EG19^T from *C. litoralis*, *H. rubra* and *H. salexigens*. *Congregibacter* forms coccoid to rod-shaped cells, only synthesizes BChl under illuminated oligotrophic conditions (Fuchs et al. 2007), lacks either any diffusible pigment or pigment absorbing at 416 nm, possesses only half the salinity tolerance of

EG19^T and lower alkalinity tolerance by 3 pH units, a narrower temperature range by 9°C, has 5.2% lower DNA G+C content, possesses cytochrome *c* oxidase activity, and is capable of utilizing propionate, sucrose and glycerol as sole carbon sources whereas EG19^T is not. Both closest related species of *Haliea* are rod-shaped and non-phototrophic, lack extracellular diffusible pigments, have considerably narrower ranges of temperature and salinity tolerance, are less tolerant to alkalinity, produce cytochrome *c* oxidase, and are unable to utilize acetate. Additionally, *H. salexigens* differs from EG19^T by surviving on glycerol, and *H. rubra* is further distinguished by being non-motile, metabolizing citrate, fructose and glucose, but not glutamate, pyruvate or succinate. On the basis of these taxonomic tests and the low 16S rRNA gene sequence similarity (95.1% to 96.2%) between EG19^T and its closest phylogenetic relatives, we propose the novel genus *Chromocurvus*, with *Chromocurvus halotolerans* as the type species.

3.5. Description of *Chromocurvus* gen. nov.

Chromocurvus (Chro.mo.cur′vus. Gr. n. *chroma*, color; L. adj. *curvus*, curved, bent; L. masc. N.L. masc. n. *Chromocurvus*, the colored curved microorganism).

Cells are pleomorphic, short to long curved rods, to irregularly shaped. Cultures are pink-purple due to the production of carotenoids and BChl *a*. Produce a LH I complex, with an absorption peak at 877 nm. No growth occurs anaerobically in the light. Obligately aerobic; no fermentation observed. The habitat of the first isolated strain is a hypersaline runoff stream from a brine spring, dominated by NaCl. Member of the *Gammaproteobacteria*. The type species is *Chromocurvus halotolerans*.

3.6. Description of *Chromocurvus halotolerans* sp. nov.

Chromocurvus halotolerans (ha.lo.to′le.rans. Gr. n. *hals* salt; L. pres. part. *tolerans* tolerating; M. L. part. adj. *halotolerans* salt-tolerating).

Exhibits the following properties in addition to those given for the genus. Cells are pleomorphic short to long curved rods to irregularly shaped (1.5-3.0 x 0.7 μm). BChl *a* gives *in vivo* absorption spectrum peaks at 802 and 877 nm, indicating the presence of RC and LH I complexes. Additional *in vivo* absorption peaks expressed at 484, 516 and 551 nm, and in acetone/methanol (7:2) extract at 467, 495 and 529 nm, are attributed to the carotenoid spirilloxanthin. A pigment expressed maximally near optimal NaCl concentration for growth exhibits absorption peaks in acetone/methanol extract at 416, 553 and 590 nm. Obligate aerobe and facultative photoheterotroph. Incapable of autotrophy. Best substrates for growth are yeast extract, bactopeptone and casamino acids; growth also occurs on acetate, glutamate, malate, pyruvate and succinate. Does not utilize butyrate, citrate, formate, glycolate, lactate, propionate, fructose, glucose, sucrose, ethanol, glycerol or methanol. When cultured on yeast extract and casamino acids, excretes an as-yet uncharacterized brownish diffusible water soluble pigment into medium, with *in vivo* absorption maxima of 230, 280 and 325 nm. Does not hydrolyse starch, Tween 60 or gelatin. Catalase positive. Cytochrome *c* oxidase negative. Optimum temperature for growth is 37°C, with growth occurring as low as 7°C and as high as 40°C. NaCl not required for growth; optimal salinity for growth is 4% NaCl, with growth occurring over a wide range of NaCl concentrations, from 0 to 18%. Optimal pH for

growth is 7.0, with growth occurring over a wide range of pH values, from 7.0 to 12.0. Resistant to a variety of antibiotics, including ampicillin, imipenem, streptomycin and kanamycin. Sensitive to chloramphenicol, nalidixic acid, penicillin G and polymyxin B. Requires biotin, thiamine and vitamin B_{12} for growth. The DNA G + C content is 63.0 mol%.

The habitat is a runoff stream from the hypersaline (56.7-66.3‰ total dissolved solids) brine spring system known as East German Creek, near Swan River, Manitoba, Canada. The type strain is EG19^T (= DSM 23344^T, = VKM B-2659^T).

3.7. Acknowledgements

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Chapter 4.

An alphaproteobacterium capable of both aerobic and anaerobic anoxygenic photosynthesis but incapable of photoautotrophy: *Charonomicrobium ambiphototrophicum*, gen. nov., sp. nov.

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Peter Schumann, and Vladimir Yurkov

The first author was the major contributor to research presented.

4.1. Abstract

A facultatively aerobic deep brown coccoid to ovoid bacterium, strain EG17^T, was isolated from a saline effluent stream in the NaCl-dominated brine spring system known as East German Creek in the province of Manitoba, Canada. The strain produced BChl a incorporated into a functional reaction center and two light harvesting complexes with absorption peaks at 802, 850 and 879 nm. EG17^T is the first reported anoxygenic phototroph capable of photoheterotrophic growth under both oxic and anoxic conditions. It yielded proportionally the greatest aerobic photosynthetic biomass under oligotrophic conditions. The results of 16S rRNA gene sequence comparisons revealed that EG17^T was related most closely to the aerobic anoxygenic phototrophs *Roseibacterium elongatum* (98.3%), and quite distantly to both *Dinoroseobacter shibae* (95.2%) and *Roseicyclus mahoneyensis* (94.7%). The DNA G + C content was 65.6 mol%. On the basis of the unique dual aerobic/anaerobic photosynthetic capability, the distinctive spectrophotometric absorption of the photosynthetic apparatus, diagnostic physiological and biochemical traits, and the moderate phylogenetic separation between EG17^T and its nearest relatives, it is concluded that this microorganism should be classified as a novel genus and species, *Charonomicrobium ambiphototrophicum* gen. nov., sp. nov., with EG17^T as the type strain.

4.2. Introduction

One of the most consistent dichotomies in microbiology is the segregation of aerobic and anaerobic photosynthesis into different species of anoxygenic phototrophs. Purple sulfur and nonsulfur bacteria (*Proteobacteria*), green sulfur bacteria (*Chlorobi*), green nonsulfur bacteria (*Chloroflexi*) and heliobacteria (*Firmicutes*) all perform photosynthesis solely in the absence of oxygen. In sharp contrast, the more recently discovered aerobic anoxygenic phototrophs (AAP) synthesize and utilize an obligately aerobically functional photosynthetic apparatus (Rathgeber *et al.*, 2004; Yurkov and Csotonyi, 2009). Possession of two photosystems (PS) allows some cyanobacteria (e.g. *Anabaena doliolum*, Bhargava *et al.*, 2008) to photosynthesize both aerobically (oxygenically, using both PS I and PS II) and anaerobically (anoxygenically, using only PS I). However, anoxygenic phototrophs, which have only a single photosystem, have never been reported to be capable of energetically harnessing light both with and without oxygen.

Strain EG17[T] is a rich brown anoxygenic phototroph that was isolated from the surface layers of a microbial mat (dominated by the marine green algae *Percursaria percursa*) in a runoff stream from a Canadian inland hypersaline spring known as East German Creek (EGC) (Csotonyi *et al.*, 2008). These thalassohaline brine springs are over 600 km from the ocean, but achieve a salinity about twice that of seawater (total dissolved solids 56.7‰ to 67.3‰) (Csotonyi *et al.*, 2008), by dissolution of salts from Devonian marine sediments (Grasby, 2000). Sulfate drives biogenic sulfide production, supporting anaerobic anoxygenic phototrophs in an illuminated zone near the surface. Aerobic mat-like growth of cyanobacteria and green algae, chiefly *P. percursa*, overlie

these communities (Csotonyi *et al.*, 2008) in streams with flow rates of nearly 4800 l/h (McKillop *et al.*, 1992). Although AAP dominate the anoxygenic phototrophic community, constituting 15-36% of cultivable bacteria, obligately anaerobic purple sulfur and nonsulfur bacteria related to *Halochromatium glycolicum* (strain EG18) and *Roseospira marina* (strain EG16), respectively, also share the habitat (Csotonyi *et al.*, 2008). This observation indicated that both aerobic and anaerobic microhabitats existed in close proximity to each other.

In this paper, we describe the first phototrophic organism possessing a single photosystem that is capable of performing both aerobic and anaerobic anoxygenic photosynthesis. The results of this research underscore the great metabolic diversity of *Proteobacteria*, and are anticipated to stimulate further study of habitats with steep oxic gradients in order to isolate and study additional representatives of this unusually plastic physiological group.

4.3. Materials and Methods

4.3.1. Isolation and cultivation

EGC was sampled in May 2002 to enumerate the anoxygenic phototrophic microbial community (Csotonyi *et al.*, 2008). EG17[T] originated from within an algal mat with 65.9‰ total dissolved solids and pH 6.9. The strain was isolated from the subsurface of an agar deep containing Medium B, designed for halophilic purple nonsulfur bacteria

(Csotonyi *et al.*, 2008), but was subsequently maintained aerobically on Medium A, for AAP (Csotonyi *et al.*, 2008).

4.3.2. Morphological, physiological and biochemical tests

Morphology was examined in late exponential-phase aerobic plate cultures on Medium A. Physiological tests for salinity (0-30%), temperature (2-45°C) and pH (4-12) tolerance, antibiotic resistance, anaerobic growth with various electron donors, aerobic and anaerobic utilization of organic carbon sources, presence of lipase, gelatinase, catalase and oxidase were determined as previously described (Yurkov *et al*., 1994). Aerobic organic carbon utilization was tested using basal carbon-free Medium A, to which the following components were added singly at a concentration of 1 g/l: acetate, arabinose, bactopeptone, casamino acids, citrate, ethanol, formate, fructose, glucose, glutamate, glycerol, glycolate, lactate, malate, methanol, propionate, pyruvate, succinate, sucrose, xylose and yeast extract. Anaerobic carbon source preference was investigated by adding singly the following carbon sources (1 g/l) to completely filled test tube cultures, using $Na_2S_2O_3$ (0.5 g/l) as electron donor: acetate, glutamate, lactate, malate, pyruvate, succinate and yeast extract. Autotrophy was tested aerobically by measuring growth in two serial transfers of liquid basal Medium A amended with 1.5 g/l $NaHCO_3$ as carbon source and 0.5 g/l $Na_2S_2O_3$ as electron donor. Anaerobic photoautotrophy was assayed in duplicate cultures of washed cells in stoppered and degassed test tubes of Medium A (4% and 8% NaCl) devoid of carbon sources except for (g/l): sodium bicarbonate (1.5) and either H_2 (headspace gas), sodium thiosulfate (0.8) or sodium sulfide (0.12) as electron

donor, a selection commonly used by purple nonsulfur bacteria. A negative control (lacking bicarbonate and electron donors) and a positive control (supplemented with 2 g/l yeast extract as carbon and electron donors) were also included.

4.3.3. Pigment analysis

Presence of BChl *a* and carotenoids was assayed spectrophotometrically in cultures that had been grown aerobically and anaerobically in liquid Medium A for 5 days. Following centrifugation, pigments were extracted from cells in acetone/methanol (7:2, v/v). Absorbance properties of pigments incorporated into the reaction center (RC) and light harvesting (LH) complexes were determined *in vivo* (cells resuspended in 0.1 M Tris-HCl buffer, pH 7.8, and 30% bovine serum albumin), using a spectrophotometer (U-2010; Hitachi), as previously described (Yurkov *et al.*, 1994).

4.3.4. Photosynthetic growth

Growth of EG17[T] was measured (determined by the Bradford Assay; Bradford, 1976) under aerobic (30 ml cultures in 125-ml flasks) and anaerobic (completely filled 10-ml test tubes) conditions in the light (500 lux) and in the dark, on Medium B+G, which was based on Medium B (Csotonyi *et al.*, 2008) but supplemented with glutamate (1 g/l) as additional carbon source, and with yeast extract content reduced to 0.1 g/l. Cultures were also grown on Medium B+G+Y, an organic rich variant of Medium B+G with 1 g/l yeast extract. Performance was compared to that of strain BF-9, a typical purple nonsulfur

bacterium closely related (98.0% by 16S rDNA) (Bilyj and Yurkov, unpublished) to *Rhodobacter capsulatus*, cultured on modified PNS medium (Bilyj and Yurkov, unpublished) for purple nonsulfur bacteria, containing (g/l): acetate (1), malate (1) and yeast extract (0.2) as carbon sources and additionally supplemented with $MgSO_4$ (0.5) as sulfur source. Cultures were incubated at a temperature of 28^oC in either a thermostatically controlled shaker at 120 cycles/minute (aerobic cultures) or in an incubator (anaerobic tubes).

4.3.5. DNA G+C content determination and 16S rRNA gene sequence analysis

The DNA G+C content was determined by using HPLC of nucleosides obtained according to Mesbah *et al*. (1989). For phylogenetic analysis, genomic DNA was extracted, and the 16S rRNA gene sequence (greater than 1400 nucleotides long) was PCR-amplified and directly sequenced as by Rainey *et al*. (1996). Sequence reaction mixtures were electrophoresed using a model 373A automatic DNA sequencer (Applied Biosystems). The sequences were aligned with published data obtained from the EMBL nucleotide sequence database and the Ribosomal Database Project, using the ae2 editor (Maidak *et al*. 1999), and similarity values were determined. A neighbor-joining dendrogram was reconstructed from a distance matrix using the treeing algorithm of Felsenstein (1993). Bootstrap values were determined according to Felsenstein (1985). The sequence accession number AM691091 was obtained for EG17[T].

4.4. Results and Discussion

4.4.1. Culture properties and morphology

Under both aerobic and anaerobic conditions, strain EG17[T] formed ~ 2-3 mm-diameter rich dark brown convex entire colonies on the surface of agar media. Anaerobic cultures were similar but more deeply pigmented, a blackish brown. Liquid aerobic cultures on Medium A were brown in color within 1 day, but in liquid Medium B+G or Medium B+G+Y, cultures were pink, achieving this color after 3 days. Anaerobic liquid cultures in Medium A or B were reddish brown, within a day of growth, similar to aerobic growth. Like its distant relative *Dinoroseobacter shibae* (Biebl *et al*., 2005), cells of strain EG17[T] were coccoid to rod-shaped (0.9 x 0.9-2.6 μm) (Fig. 4.1), but several times as large as cells of *D. shibae* (0.3-0.7 x 0.3-1.0). There was an even greater difference between the morphology of EG17[T] and its nearest phylogenetic neighbours, the long narrow (0.5-0.8 x 1.6-10 μm) rod-shaped *Roseibacterium elongatum* (Suzuki *et al*., 2006), and *Roseicyclus mahoneyensis*, which exhibits a strongly curved, almost cyclical shape (Rathgeber *et al*, 2005).

4.4.2. Photosynthetic apparatus and pigmentation

In vivo suspensions of cells absorbed light maximally at 589, 802 and 879 nm (and additionally at 850 nm anaerobically) indicating the presence of BChl *a* (confirmed by
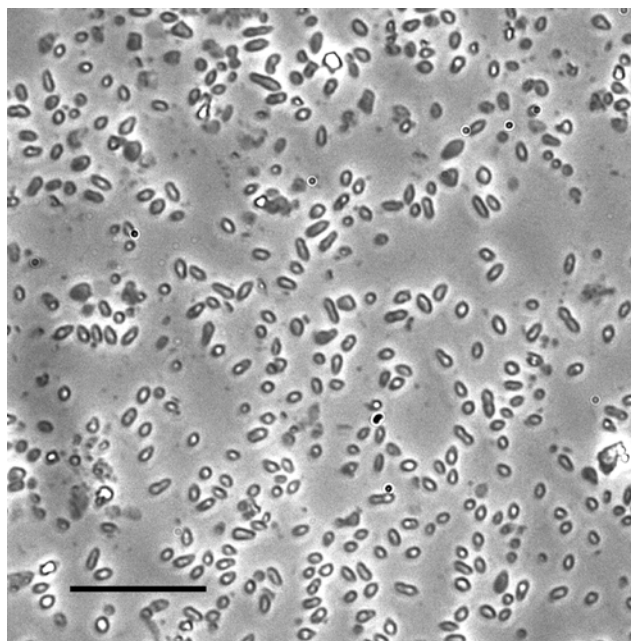
Fig. 4.1. Phase contrast micrograph of coccoid to rod-shaped cells of strain EG17$^\mathrm{T}$. Scale

bar, 10 µm.
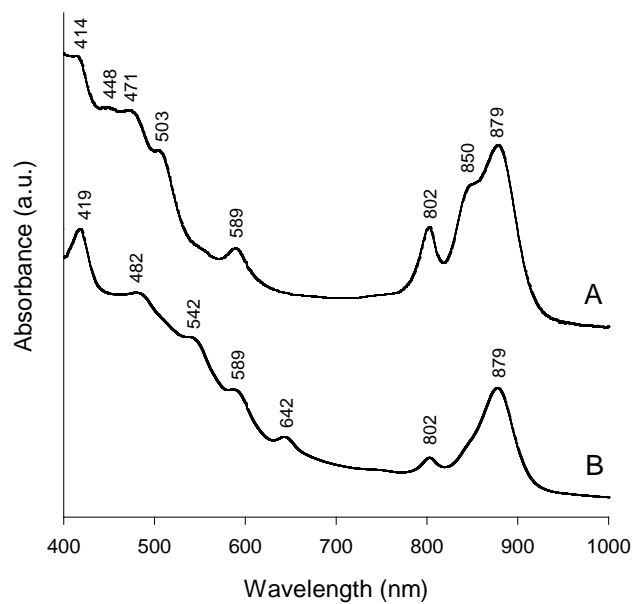
Fig. 4.2. Photosynthetic apparatus and pigmentation in EG17[T]. *In vivo* absorption spectrum. **(A)** Anaerobically grown cells. **(B)** Aerobic culture. Numerals above spectra denote λ_{max} values (nm).

λ_{max} at 770 nm in acetone/methanol [7:2] extract) incorporated into typical purple

bacterial RC and two LH complexes (Fig. 4.2). The 802 and 850 nm peaks indicated the

presence of a typical *Rhodobacter*-type LHII that was best expressed under anaerobic

conditions (Fig. 4.2a).

EG17[T] The photosynthetic apparatus of EG17[T] stood substantially apart from all of its

nearest relatives, most notably because it was expressed similarly well anaerobically and

aerobically (unlike any of its obligately AAP phylogenetic affiliates, *R. elongatum*, *D.*

shibae and *R. mahoneyensis*). Important spectrophotometric characteristics also set apart

the photosynthetic apparatus of EG17[T]. Of its closer relatives, *R. mahoneyensis* and *D.*

shibae have a second LH complex. However, they both appear to possess unusual

monomodal LHII complexes (λ_{max} = 806 nm for *R. mahoneyensis* and λ_{max} = 804 nm for

D. shibae) (Biebl *et al.*, 2005; Rathgeber *et al.*, 2005), unlike that of the bimodal LHII of

EG17[T] (λ_{max} = 802, 850 nm) (Fig. 4.2). Furthermore, the LHI of EG17[T] (λ_{max} = 879 nm)

was red-shifted by 8-9 nm compared to that of *R. mahoneyensis* (λ_{max} = 870-871 nm),

and by 11 nm compared to *D. shibae* (λ_{max} = 868 nm) (Table 4.1), which indicated a

distinctly different protein environment of this LHI complex. It was most similar to the

879 nm LHI of its nearest relative, *R. elongatum*, a species which nevertheless has only a

single LH complex (Suzuki *et al.*, 2006).

EG17[T] produced prodigious photosynthetic pigments (BChl and carotenoids)

under both aerobic (dark and illuminated) and anaerobic (illuminated) conditions (Fig.

4.2). This was unusual because oxygen typically inhibits pigment expression in anaerobic

purple phototrophs growing aerobically by strict heterotrophy. Conspicuous exceptions

are the purple nonsulfur bacteria *Rhodocista centenaria* and *Rhodovulum sulfidophilum*,

Table 4.1. Distinguishing phenotypic traits of *Charonomicrobium ambiphototrophicum* EG17^T in comparison with other phylogenetic relatives. Species: 1, EG17^T; 2, *Roseibacterium elongatum* OCh-323^T; 3, *Dinoroseobacter shibae* DFL 12^T; 4, *Roseicyclus mahoneyensis* ML6^T. -, negative; (+), weak positive; +, positive; ++ strongly positive; NA, not available.

Characteristic	1	2[1]	3[2]	4[3]
Isolation source	Canadian EGC saline spring runoff stream	Sand from Monkey Mia, Shark Bay, Australia	Cell culture of North Sea dinoflagellate *Prorocentrum lima*	Canadian meromictic Mahoney Lake
Growth characteristics				
NaCl range (%)	2-16	0.5-7.5	1-7	0.5-10
NaCl optimum (%)	8-12	NA	NA	NA
pH range	7-10	NA	6.2-9.2	6-11
pH optimum	7-8	7.5-8	6.5-8.8	6-11
Temp. range (°C)	7-37	NA	15-38	10-37
Temp. optimum (°C)	28-37	27-30	33	28
Anaerobic growth	+	-	-	-
Autotrophic growth	-	-	-	-
Nitrate reduction	(+)	-	+	-
Catalase	+	+	+	+
Oxidase	-	+	+	+
Pigmentation				
BChl *a in vivo* peaks (nm)	802, 850, 879	800, 879	804, 868	805-806, 870-871
Aerobic utilization of:				
Acetate	(+)	-	+	+
Citrate	(+)	-	+	+
Formate	-	NA	NA	-
Glutamate	(+)	NA	+	+
Lactate	(+)	NA	+	+
Malate	-	-	+	+
Propionate	-	NA	NA	NA
Pyruvate	(+)	-	+	+
Succinate	-	-	+	+
Arabinose	-	NA	NA	NA
Fructose	(+)	NA	+	++
Glucose	(+)	-	+	++
Maltose	(+)	NA	NA	NA
Sucrose	-	NA	NA	NA
Xylose	-	NA	NA	NA
Ethanol	-	-	-	-
Glycerol	-	NA	+	NA
Methanol	-	NA	-	NA
Bactopeptone	(+)	NA	NA	NA
Casamino acids	-	NA	NA	NA
Yeast extract	++	NA	+	++
Hydrolysis of:				
Starch	-	-	-	+
Gelatin	-	+	+	+
Tween 60	(+)	NA	NA	-

Antibiotic sensitivity:

Ampicillin	+	NA	NA	-
Chloramphenicol	+	NA	+	NA
Imipenem	+	NA	NA	NA
Kanamycin	+	NA	NA	+
Nalidixic acid	(+)	NA	NA	-
Penicillin G	+	NA	+	-
Polymyxin B	+	NA	+	+
Streptomycin	+	NA	NA	+
Acid production from:				
Fructose	(+)	-	NA	NA
Glucose	(+)	-	-	NA
Sucrose	(+)	NA	NA	NA

[1] Data from Suzuki *et al.* (2006).
[2] Data from Biebl *et al.* (2005).
[3] Data from Rathgeber *et al.* (2005).

in which photosynthesis genes are repressed only slightly aerobically, but which are nevertheless only capable of photosynthesizing anaerobically. Light generally inhibits BChl synthesis aerobically in AAP (Yurkov and Csotonyi, 2009). For example, the AAP *Roseobacter denitrificans* produced no BChl under illumination of 1.2 W/m^2, or ~800 lux (Shioi and Doi, 1988). Under illuminated (~600 lux) aerobic conditions, EG17[T] produced 65% as much BChl as in complete darkness. By comparison, light completely inhibits BChl production in *R. mahoneyensis* (Rathgeber *et al*., 2005). The difference between light and dark BChl formation was not evaluated for *R. elongatum*. However, a dark chemostat culture of *D. shibae* produced BChl at a rate about 15% as high as in the light (1400 lux) (Biebl and Wagner-Döbler, 2006), making *D. shibae* a somewhat atypical AAP. On the whole, pigment synthesis in EG17[T] was more like that of purple nonsulfur bacteria with respect to illumination, but it resembled that of typical AAP with respect to aeration.

Additional pigments, mainly carotenoids, absorbed *in vivo* at 414, 448, 471, 503 and 549 nm (anaerobically), and at 419, 482, 542 and 642 nm (aerobically) (Fig. 4.2). The marked difference in absorbance spectra under oxic and anoxic conditions suggests that EG17[T] produced a different suite of carotenoids under each condition, much as *R. sphaeroides* produces only spheroidene under anaerobic conditions but synthesizes the oxidized form, spheroidenone, aerobically (Shneour 1962; Züllig, 1986). In fact, some of the anaerobic absorbance peaks are a near match with the *in vivo* spheroidene peaks of *R. sphaeroides* (λ_{max} = 447, 475, 507 nm) (van Dorssen *et al*., 1987). *D. shibae* possesses spheroidenone (λ_{max} = 482, 515 nm) as the only carotenoid (Biebl *et al*., 2005). The similarity between these absorption peaks and some of those of EG17[T] under aerobic

conditions suggests that EG17^T expressed a carotenoid closely related to spheroidenone. *R. mahoneyensis* also displays a quite similar aerobic absorption spectrum in the 400-550 nm region, with λ_{max} = 484 (Rathgeber *et al.*, 2005). None of the relatives of EG17^T exhibited the characteristic 642-nm component (Fig. 4.2), the identity of which is unknown. A few components absorb at this wavelength, such as cytochrome *bd*. This heme is widely distributed in the *Bacteria* and *Archaea* (Winstedt *et al.* 1998), and it has an important role in microaerobic nitrogen fixation in the enteric bacterium *Klebsiella pneumoniae*, where it is expressed under all conditions that permit diazotrophy (Juty *et al.* 1997). Strong absorbance of blue light at 414 or 419 nm was most likely due to cytochromes as well, and a similar characteristic is observed in *D. shibae* (λ_{max} = 410) (Biebl *et al.*, 2005) and *R. mahoneyensis* (λ_{max} = 408) (Rathgeber *et al.*, 2005).

4.4.3. Aerobic and anaerobic photosynthetic growth

Growth occurred anaerobically in the light and aerobically in either illuminated or dark conditions; light was not required aerobically, but no anaerobic growth occurred in its absence. This combination of physiological characters would classify EG17^T as a purple nonsulfur bacterium. However, its capacity to grow more rapidly under illuminated than dark conditions aerobically was anomalous. Notably, none of its close phylogenetic phototrophic relatives are capable of anaerobic growth; *R. elongatum*, *D. shibae* and *R. mahoneyensis* are all AAP. Although AAP are frequently closely affiliated with non-phototrophic heterotrophs (Rathgeber *et al.*, 2004; Yurkov and Csotonyi, 2003, 2009), it is interesting that all of the closest relatives of this purple nonsulfur bacterium are AAP.

This topological peculiarity of its phylogeny may simply be due to the fact that no other members of this particular phylogenetic clade of phototrophs have yet been isolated; only time and sampling effort will be able to confirm this.

Strain EG17[T] was capable of anaerobic photosynthesis (Fig. 4.3a), exhibiting a growth curve typical of anaerobic purple nonsulfur bacteria such as *R. capsulatus*-relative BF-9 (Fig. 4.3b) in the presence of light and absence of oxygen. It could not grow in the dark anaerobically. Unexpectedly, EG17[T] also possessed the ability to harness light energy aerobically, which has not yet been demonstrated for any anaerobic anoxygenic phototroph. Three lines of evidence support this conclusion.

First, when cultured in aerobic oligotrophic (0.1 g/l yeast extract) conditions, EG17[T] accumulated about 3 times as much biomass (and at a rate about 3.5 times as high) in the presence of light as in its absence (Fig. 4.3c). Under nearly similar conditions (0.2 g/l yeast extract), the typical purple nonsulfur BF-9 did not yield more biomass in light versus dark aerobic culture (Fig. 4.3d). Although maximal growth rates were similar between light and dark treatments in eutrophic culture (1 g/l yeast extract), illuminated EG17[T] cultures ultimately yielded about 39% more biomass than dark cultures, even under these carbon-rich conditions (Fig. 4.3c).

A second indicator of the aerobic photosynthetic activity of EG17[T] was the apparent paradox that in eutrophic culture, light actually retarded growth initially (Fig. 4.3c). This observation is only counterintuitive until it is considered that in AAP heterotrophic and photosynthetic energy conservation pathways share electron carriers (Yurkov and Beatty, 1998; Beatty, 2002). It is unknown how photosynthesis and
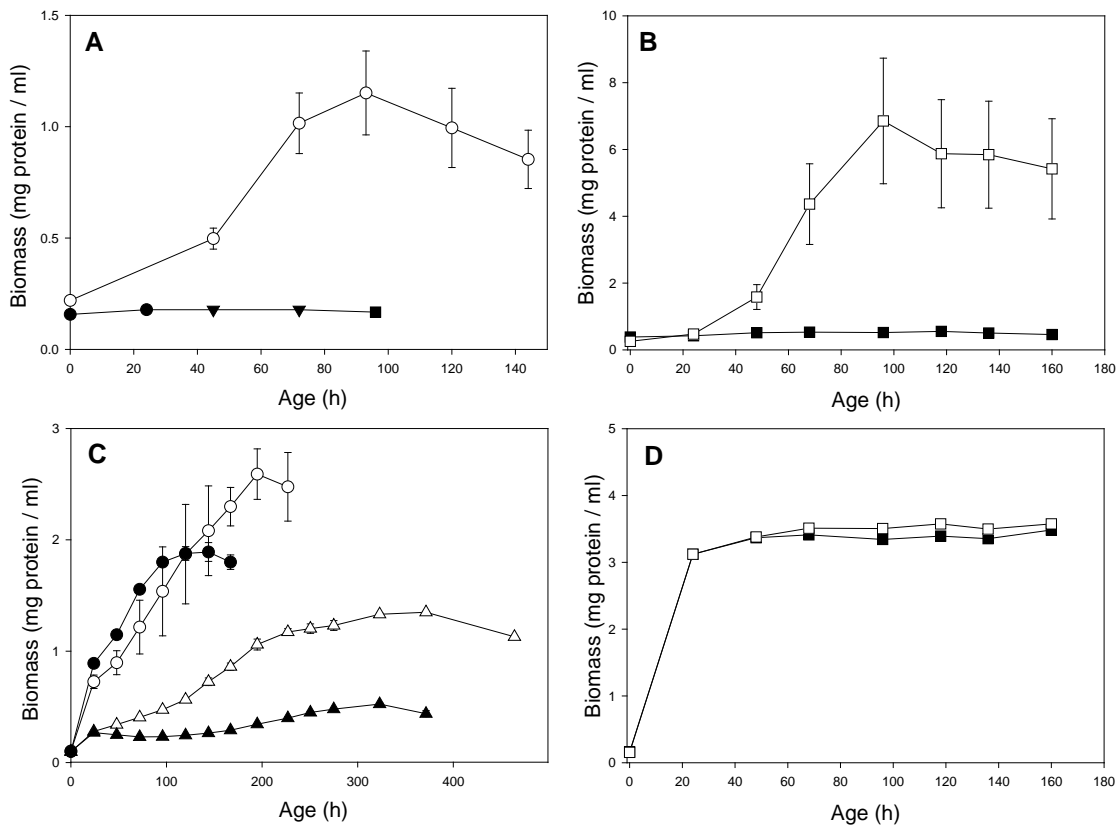
Fig. 4.3. Aerobic and anaerobic photosynthetic and heterotrophic growth of EG17^T compared to typical purple nonsulfur bacterium BF-9. **(A)** Anaerobic cultures of EG17^T on basal Medium B with 1 g/l yeast extract as sole carbon source, with (–○–) and without (–●–) light. **(B)** Anaerobic cultures of BF-9 on PNS medium in light (–□–) and dark (–■–). **(C)** Aerobic cultures of EG17^T on Medium B+G+Y (eutrophic, 1 g/l yeast extract) (symbols as in A) and on Medium B+G (oligotrophic, 0.1 g/l yeast extract), illuminated (–△–) and dark (–▲–). **(D)** Aerobic cultures of BF-9 (symbols as in B). (A), (C), means and standard errors from duplicate cultures; (B), (D), means and standard errors from triplicate cultures.

respiration can coexist in AAP, but respiration appears to compete with photosynthesis for rate-limiting functional components, a situation that is avoided in purple nonsulfur bacteria by the repression of photosynthesis when O_2 is available for heterotrophic growth (Bauer *et al*., 2009). If photosynthesis (a last-resort physiological adaptation that yields the greatest advantage under severely carbon-limited conditions) is not repressed by respiration and generates slower growth than the highly opportunistic tricarboxylic acid cycle, then exposure to light should sequester some of the electron carriers and reduce the rate of chemotrophic carbon utilization when both forms of energy are available. However, light can amend the energy needs of AAP by 20-30% (Yurkov and Csotonyi, 2009). It should therefore preclude the need to use reduced carbon for all of the cell's energy requirements, shunting some carbon from a catabolic to an anabolic role, and resulting in higher ultimate biomass yield. This overall pattern is exactly what was observed in EG17[T] under eutrophic conditions: slower initial growth but a 39% higher ultimate biomass yield under illumination (Fig. 4.3c). By contrast, growth of the purple nonsulfur bacterium BF-9 showed no such trend, as growth rates and final yields were similar with or without light (Fig. 4.3d).

Third, under oligotrophic conditions, after consumption of the easily metabolized yeast extract, illuminated EG17[T] commenced growth at a slower rate, on one of the less preferred organic carbon sources, acetate or glutamate. This occurred at 24 hours, when dark-grown aerobic EG17[T] cultures reached a slower growth phase with just over 1/10 the biomass yield of eutrophic cultures that were amended with 10 times as much yeast extract. Photoheterotrophic efficiency on acetate and glutamate was much higher than was the chemoheterotrophic use of these substrates: illuminated cultures generated over

five times as much biomass in this second phase of growth as did dark cultures. This observation further confirmed that EG17^T was photosynthetically active aerobically.

The relative light-insensitivity of BChl synthesis in EG17^T is crucial to its high photosynthetic metabolic efficiency. AAP generally produce BChl only at night (Yurkov and Beatty, 1998; Yurkov and Csotonyi, 2009), when light-induced aerobic conversion of BChl into a toxic triplet state is avoided (Beatty, 2002). This strategy, however, limits their ability to utilize light for energy because cellular pools of BChl decline during the day as cells divide, especially in high latitude summers where days are twice or more as long as nights. By producing 65% as much BChl under continuous light aerobically as in complete darkness, EG17^T maximizes its photosynthetic energy return. Experiments with marine AAP such as *Dinoroseobacter shibae* reveal that when the light:dark regimen is changed from 8h:16h (winter conditions) to 16h:8h (summer conditions), the nightly rate of BChl synthesis quadruples (Biebl and Wagner-Döbler, 2006). Further investigation with EG17^T should address whether its physiology is similarly optimized for high latitudes.

Like all of its close AAP relatives, strain EG17^T was incapable of aerobic autotrophic growth, which is typical for this group. However, EG17^T was also incapable of anaerobic photoautotrophy, when supplemented with hydrogen, thiosulfate (0.8 g/l) or sulfide (0.12 g/l) as electron donor. Either EG17^T does not possess genes for the Calvin cycle, or it has non-functional genes or promoter for the biochemical pathway. The absence of photoautotrophy sets this organism apart from all known anaerobic phototrophic *Proteobacteria* and all anaerobic anoxygenic species except the obligately photoheterotrophic gram-positive heliobacteria. Thus far, the presence of the Calvin cycle

has been a reliable distinction between anaerobic purple bacteria and AAP, because all species of the former group express it, whereas none of the latter do so. A notable partial exception is the purple nonsulfur bacterium *Rhodopseudomonas cryptolactis*, which is incapable of strict photoautotrophy (Stadtwald-Demchick *et al*., 1990). However, although the species is also unable to metabolize lactate alone, it can survive on a combination of lactate and bicarbonate, which the authors interpreted as evidence that the Calvin cycle is used as an electron sink for the oxidative conversion of lactate to pyruvate, on which *R. cryptolactis* can grow (Stadtwald-Demchick *et al*., 1990). Sequencing will be required to verify the presence or absence of genes of the Calvin cycle in EG17^T. Their absence, along with the dual aerobic/anaerobic photosynthetic capability would implicate EG17^T as a true physiological/evolutionary intermediate between purple nonsulfur bacteria and AAP.

4.4.4. Biochemical and physiological characteristics

Physiological characteristics of strain EG17^T are presented in Table 4.1. Strain EG17^T grew over a markedly greater range of salinity than any of its closest relatives, thriving between 4 and 14% NaCl, and showing some growth at 2 and 16% NaCl. The meromictic lake dwelling *R. mahoneyensis* comes closest to this range, growing at 0.5 to 10% NaCl (Rathgeber *et al*., 2005). EG17^T has a higher minimum salt concentration requirement as well: 2%, compared with 0.5% for *R. elongatum* (Suzuki *et al*., 2006) and *R. mahoneyensis* (Rathgeber *et al*., 2005), and 1% for *D. shibae* (Biebl *et al*., 2005). The optimum salinity for growth, occurring at 8 to 12% NaCl, reflects the high salinity

requirement (Table 4.1). In general, therefore, EG17^T is better adapted to a hypersaline and osmotically fluctuating environment than are even the meromictic lake inhabitant *R. mahoneyensis* (Rathgeber *et al*., 2005), the marine *D. shibae* (Biebl *et al*., 2005) or the marine intertidal *R. elongatum* (Suzuki *et al*., 2006). This trend is supported by the observation that, at any given time, the EGC aquatic habitat of EG17^T ranges in salinity from a stream center value of 65.9‰ to saturation at some nearby soil surfaces (Csotonyi *et al*., 2008). However, these values may be still further extended by precipitation, temporarily lowering the salinity, and by salinity-elevating desiccation during especially hot weather or low stream flow.

Optimal pH for growth of EG17^T was 7.0 to 8.0 (Table 4.1), reflecting the neutral (pH 6.9) source stream of EGC (Csotonyi *et al*., 2008). It tolerated pH up to 10.0. This range is quite typical of anoxygenic phototrophs from some other saline environments (Yurkova *et al*., 2002), such as *R. mahoneyensis* (Rathgeber *et al*., 2005).

EG17^T grew under a wide range of temperatures, from 7°C to 37°C, reflecting the lack of an insulating cover of vegetation on the salt flats of EGC (Csotonyi *et al*., 2008). This likely causes wide swings in temperature in the summer (when cells must tolerate intermittent peaks of high temperature), and in the spring and fall (when the paucity of vegetation might fail to ameliorate the effects of cold air masses). It may be why the range of temperature over which EG17^T grows is wider than that of any of its nearest relatives (Table 4.1).

Under dark aerobic heterotrophic conditions, EG17^T strongly preferred yeast extract as an organic carbon source, and growth was very weak on acetate, citrate, glutamate, glycolate, lactate and pyruvate; the carbohydrates fructose, glucose and

maltose; the complex organic bactopeptone (Table 4.1). In its strong preference for yeast extract, EG17[T] resembled its AAP relative *R. mahoneyensis* (Rathgeber *et al.*, 2005). In fact, like *R. mahoneyensis*, EG17[T] appeared to require an unknown growth factor present in yeast extract, as there was no appreciable growth in its complete absence; a mixture of biotin, vitamin B_{12}, nicotinic acid and thiamine could not replace yeast extract as a required component. However, EG17[T] differed from *R. mahoneyensis* in being incapable of utilizing two of the carbon sources that this species can metabolize, namely malate and succinate. Unlike *R. elongatum*, EG17[T] could grow weakly with acetate, citrate, pyruvate and glucose. EG17[T] and *D. shibae* differed in the ability of the latter to make use of malate, succinate and glycerol (Table 4.1). Anaerobically in the light, EG17[T] grew extremely weakly with acetate, glutamate, lactate, malate, and succinate, and it grew strongly only with yeast extract as a carbon source. The fact that only the complex carbon source yeast extract supported strong anaerobic growth of EG17[T] out of a set of carbon sources used by the majority of purple nonsulfur bacteria made EG17[T] unusual. Only a minority of purple nonsulfur bacteria, including *Rhodopseudomonas cryptolactis*, display such restricted diversity of anaerobic carbon utilization. The results suggest that EG17[T] derives from its highly organic rich environment of EGC a specific and reliable carbon source.

EG17[T] was unusual in displaying no cytochrome *c* oxidase activity. Most aerobic bacteria that respire with oxygen, including *R. elongatum*, *R. mahoneyensis* and *D. shibae*, possess this enzyme. EG17[T] was in this regard similar to members of the distantly related genus *Octadecabacter* (Gosink *et al.* 1997), aerobic bacteria that, like EG17[T], grow very poorly on defined carbon sources (Gosink *et al.* 1997). Only very weak acid

production on glucose (pH 6.6), sucrose (pH 6.5) and fructose (pH 6.3) was observed (Table 4.1), which does not constitute fermentation. Among its relatives, neither *R. elongatum* nor *D. shibae* displayed any fermentative ability. This is as expected, for AAP are not known to ferment. EG17[T] was capable of weak nitrate reduction to nitrite, distinguishing it from *R. elongatum* and *R. mahoneyensis*, which are incapable of dissimilatory nitrate reduction (Rathgeber *et al*., 2005; Suzuki *et al*., 2006), and from *D. shibae*, which reduces nitrate to a more reduced form than nitrite, but without a nitrite intermediate (Biebl *et al*., 2005). Unlike *R. mahoneyensis*, EG17[T] hydrolyzed Tween 60 but not starch, and unlike any of its nearest relatives, it was incapable of gelatin hydrolysis (Table 4.1). EG17[T] was sensitive to all eight antibiotics tested. This is rather high sensitivity compared to some α-4 proteobacterial AAP, such as *Porpyrobacter meromictius* (Rathgeber *et al*., 2007). It also differed from the nearer relative *R. mahoneyensis*, which resists ampicillin, nalidixic acid and penicillin G (Rathgeber *et al*., 2005) (Table 4.1).

4.4.5. DNA composition and phylogenetic analysis

The analysis of nearly-complete 16S rRNA gene sequences (> 1400 nucleotides) revealed that strain EG17[T] was a member of the *Alpha-3-Proteobacteria* and defined a novel lineage (Fig. 4.4). The nearest relatives according to sequence similarity were the AAP species *R. elongatum* (98.3%), *D. shibae* (95.2%) and *R. mahoneyensis* (94.7%). The 16S rRNA gene sequence of EG17[T] was only 93.0% and 93.7% similar to the purple nonsulfur bacteria *Rhodobacter capsulatus* and *Rhodovulum adriaticum*, respectively
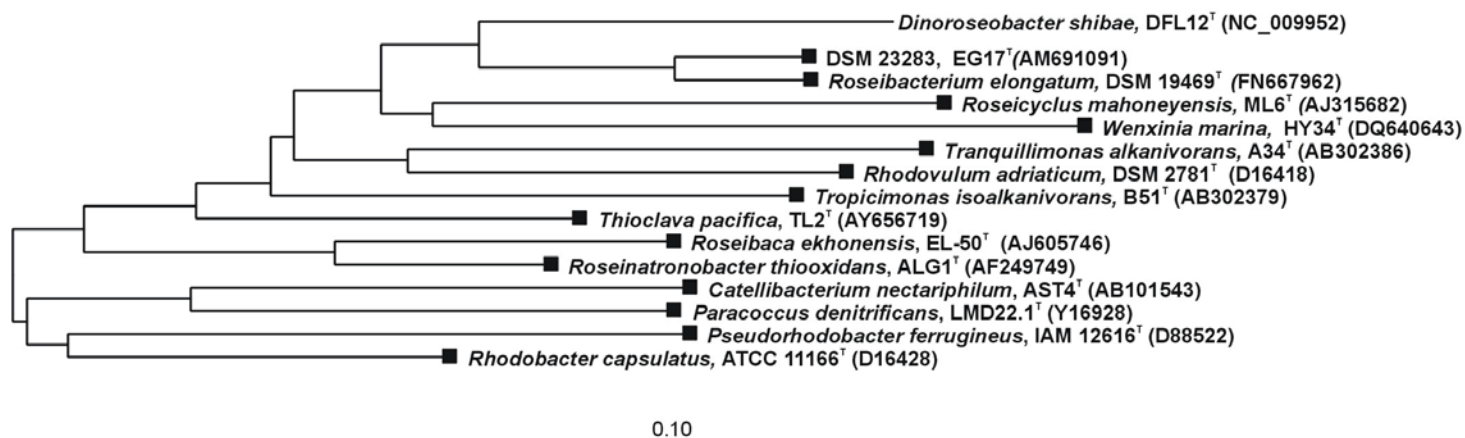
Fig. 4.4. Neighbour-joining dendrogram of 16S rRNA gene sequence relatedness, showing the position of *Charonomicrobium ambiphototrophicus* strain EG17^T and its phylogenetic neighbours, i.e. the AAP genera *Roseibacterium*, *Dinoroseobacter*, *Roseicyclus* and other members of the *Roseobacter* clade, α-3 cluster of the *Proteobacteria*. Tree was rooted with 16S rRNA gene sequences of other members of the *Alphaproteobacteria*. Bar, 10 substitutions per 100 sequence positions.

(Fig. 4.4). Hence, its closest relatives thus far isolated are AAP, not purple nonsulfur bacteria. The above phylogenetic distances are generally sufficient to distinguish between different genera, even aside from the difference in the possession of major physiological capabilities (anaerobic photosynthesis and autotrophy), and such metabolic capacities are traditionally used as genus-level distinguishing characteristics (Stackebrandt *et al*., 2002).

The DNA G + C content of EG17[T] was 65.6 mol%, which different slightly from the nearest phylogenetic affiliates of the organism: 68.1 mol% for *R. elongatum* (Suzuki *et al*., 2006), 64.8 mol% for *D. shibae* (Biebl *et al*., 2005) and 66.2 mol% for *R. mahoneyensis* (Rathgeber *et al*., 2005).

In summary, photosynthesis first arose in the reducing environment of the early earth, so it was adapted to function anaerobically, a hallmark of heliobacteria, purple and green sulfur and nonsulfur bacteria even today. However, after over two billion years of evolution since the oxygenation of the atmosphere by cyanobacteria, the apparent complete absence of an organism possessing a photosynthetic system with appreciable redox flexibility would be peculiar. It is particularly surprising because the specialized group of the AAP have evolved (presumably from purple phototrophic ancestors) to exploit exclusively aerobic niches, and they cannot photosynthesize anaerobically (Yurkov and Csotonyi, 2009). Photosystem expression in purple nonsulfur bacteria is repressed by oxygen, which in *Rhodobacter capsulatus* is facilitated aerobically by cooperative binding of the regulatory proteins CrtJ and AerR to conserved palindromic sequences in the promoters of BChl (*bch*), carotenoid (*crt*) and LHII (*puc*) genes (Masuda *et al*., 2008). Notable exceptions to this general pattern of oxic repression exist in *R*.

centenaria and *R. sulfidophilum*, purple nonsulfur bacteria that produce prodigious pigments even aerobically (Masuda *et al.*, 2008). In *R. centenaria*, AerR is structurally modified and appears to actually promote photosynthetic apparatus expression in the presence of oxygen. What function this serves in the organism, if any, is uncertain, for its photosynthetic apparatus still requires anaerobic conditions to operate. However, because homologous genes to *crtJ* and *aerR* are widely distributed among the *Proteobacteria* (Masuda *et al.*, 2008), they may be important in granting the AAP the ability to express their photosynthetic pigments aerobically. Why AAP cannot perform anaerobic photosynthesis is still debated, but the leading hypothesis concerns the possession of a primary photosynthetic electron acceptor (Q_A) with a high midpoint potential (Rathgeber *et al.*, 2004; Yurkov and Beatty, 1998; Yurkov and Csotonyi, 2009). The closest that AAP come to anaerobic photosynthesis is microaerophilic activity. For example, the gammaproteobacterial AAP *Congregibacter litoralis* is chemotactic to 10% O_2 saturation (Fuchs *et al.*, 2007); the photosynthetic apparatus of *R. mahoneyensis* is most active under microaerophilic conditions (Rathgeber and Yurkov, unpublished); maximal BChl synthesis in *Roseateles depolymerans* occurs at an atmospheric O_2 content of only 2% (Suyama *et al.*, 2002); and *Roseinatronobacter thiooxidans* best synthesizes BChl microaerophilically (Sorokin *et al.*, 2000).

The ability of EG17[T] to synthesize and use a photosynthetic apparatus under anoxic conditions sets it apart from AAP, as does its LHII complex, which is reminiscent of purple nonsulfur bacteria, for the B850 LHII has not yet been found in AAP (Yurkov and Beatty, 1998; Rathgeber *et al.*, 2004; Yurkov and Csotonyi, 2003, 2009). However, the aerobic photosynthetic capability of EG17[T] also distinguishes it from purple

nonsulfur bacteria. The unique dual redox nature of its mode of photosynthesis suggests that EG17^T is an intermediate between AAP and their purple nonsulfur bacterial ancestors, a sort of 'missing link'. Its incapacity for autotrophy further underscores this idea. Dual aerobic/anaerobic phototrophy in EG17^T is best suited to an illuminated turbulent habitat with a steep gradient in redox potential over a short distance, where cells are readily transported between these microenvironments. It is therefore not surprising that such an organism was first isolated from a sulfide-supplied lotic habitat such as EGC. Future studies will need to investigate (1) whether the genes for the Calvin cycle are present in EG17^T, and (2) the biophysics of this organism's photosynthetic apparatus to elucidate the characteristics that allow it to promote functional electron flow under such a wide range of reduction potentials. Recent discovery of the simultaneous presence of two photosynthetic operons (one from *Alphaproteobacteria* and one from *Gammaproteobacteria*) in JL354, a close relative of *Citromicrobium bathyomarinum* (Jiao *et al*., 2010) should also stimulate photosynthetic gene sequencing of EG17^T to determine whether it accomplishes aerobic and anaerobic phototrophy using two independent photosynthetic apparatuses.

Phylogenetic analysis demonstrated that EG17^T was relatively distantly related to the photosynthetic genera *Roseibacterium*, *Dinoroseobacter* and *Roseicyclus*. Physiological and biochemical differences further supported the distinction of EG17^T from them. In particular, capacity for both anaerobic and aerobic photoorganotrophic growth serves as a sufficiently distinctive marker to prompt the establishment of a novel genus, for it delineates a major physiological capability that no other anoxygenic phototroph has yet been demonstrated to possess. Furthermore, EG17^T possessed two LH

complexes that were unlike either the single LH complex of *R. elongatum*, or the two LH complexes of *R. mahoneyensis* and *D. shibae*. Its coccoid to rod-shaped morphology and cell size, high tolerance and preference for salinity, absence of cytochrome *c* oxidase and distinct patterns in the metabolism of gelatin, starch, Tween 60, nitrate and seven reduced carbon sources also set EG17^T apart from its nearest phylogenetic neighbours.

On the basis of these taxonomic markers and the sufficiently low 16S rRNA gene sequence similarity (94.7-98.3%) between the novel isolate and its closest phylogenetic relatives, we propose the novel species *Charonomicrobium ambiphototrophicum*.

4.5. Description of *Charonomicrobium* gen. nov.

Charonomicrobium (Cha.ro′.no.mic.ro′.bi.um. Gr. n. *Charon* boatswain of Styx River in Greek and Roman mythology, the only being who could cross regularly between the common world and the underworld, symbolizing the way in which EG17^T is able to survive by photosynthesizing on both sides of the aerobic/anaerobic redox gradient; N.L. neut. n. *microbium,* a microbe; N.L. n. *Charonomicrobium*, the Charon microbe).

Cells are coccoid to rod-shaped. Cultures are rich deep brown due to the production of carotenoids and BChl *a*. Produce LHI complex (expressed aerobically and anaerobically), with an absorption peak at 879 nm, and LHII complex (expressed mostly anaerobically), with absorption peaks at 802 and 850 nm. Growth occurs photoorganotrophically both aerobically and anaerobically in the light. Facultatively aerobic. The habitat of the first isolated strain is a hypersaline runoff stream from a brine spring, dominated by NaCl.

Member of the *Alphaproteobacteria*. The type species is *Charonomicrobium ambiphototrophicum*.

4.6. Description of *Charonomicrobium ambiphototrophicum* sp. nov.

Charonomicrobium ambiphototrophicum (am′.bi.pho.to.tro′.phi.cum. Gr. adj. *ambi* both; Gr. n. *phos photos* light; Gr. adj. *trophikos* nursing, tending or feeding; N.L. adj. *ambiphototrophicum* referring to the ability to use light in both modes of energy generation, aerobic and anaerobic.)

Exhibits the following properties in addition to those given for the genus. Cells are coccoid to rod-shaped (0.9 x 0.9-2.6 µm). BChl *a* gives *in vivo* absorption spectrum peaks at 589 nm, 802 nm, 850 nm (indicating an LHII complex expressed mainly anaerobically) and 879 nm (an LHI expressed aerobically and anaerobically). Additional *in vivo* absorption peaks expressed at 414, 448, 471, 503 and 549 nm (anaerobically), and at 419, 482, 542 and 642 nm (aerobically) correspond mainly to carotenoids. Aerobic organoheterotroph and facultatively aerobic photoheterotroph, capable of photosynthesis aerobically and anaerobically. Incapable of autotrophy, either aerobically or anaerobically. Catalase positive. Oxidase negative. Best substrate for growth is yeast extract (for anaerobic photoheterotrophy and aerobic organoheterotrophy). Yeast extract required as growth factor. Weak aerobic growth also occurs with acetate, citrate, glutamate, glycolate, lactate, pyruvate, fructose, glucose, maltose and bactopeptone. Aerobic photoheterotrophic growth occurs on acetate and glutamate. Does not utilize

formate, malate, propionate, succinate, arabinose, sucrose, xylose, ethanol, glycerol, methanol and casamino acids. Weak acid production from carbohydrates observed. Weak dissimilatory reduction of nitrate to nitrite observed. Tween 60 is hydrolyzed but starch and gelatin are not. Optimum salinity for growth is 8-12% NaCl, with growth occurring over the range of 2 to 16% NaCl. Sensitive to ampicillin, chloramphenicol, imipenem, kanamycin, nalidixic acid, penicillin G, polymyxin B and streptomycin. Optimum temperature for growth is between 28 and 37°C, with growth occurring at temperatures as low as 7°C and as high as 37°C. Optimal pH for growth is 7.0-8.0, with growth occurring over a range of pH values, from pH 7.0 to 10.0. The DNA G + C content is 65.6 mol%.

The habitat of the first isolated strain is a green algae-rich runoff stream from a thalassohaline hypersaline spring system (65.9‰ total dissolved solids and pH 6.9) with a subterranean source of sulfide (52° 45′ 10 ″ N, 100° 52′ 50″ W). The type strain is EG17^{T}.

4.7. Acknowledgements

4.8. References

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Chapter 5.

Anaerobic respiration on tellurate and other metalloids in bacteria

from hydrothermal vent fields in the Eastern Pacific Ocean.

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The first author was the major contributor to research presented.

5.1. ABSTRACT

This paper reports the discovery of anaerobic respiration on tellurate by bacteria isolated from deep ocean (1543-1791 m) hydrothermal vent worms. The first evidence for selenite- and vanadate-respiring bacteria from deep ocean hydrothermal vents is also presented. Enumeration of the anaerobic metal(loid)-resistant microbial community associated with hydrothermal vent animals indicates that a greater proportion of the bacterial community associated with certain vent fauna resists and reduces metal(loid)s anaerobically than aerobically, suggesting that anaerobic metal(loid) respiration might be an important process in bacteria that are symbiotic with vent fauna. Isolates from Axial Volcano and Explorer Ridge were tested for their ability to reduce tellurate, selenite, metavanadate or orthovanadate in the absence of alternate electron acceptors. In the presence of metal(loid)s, strains showed an ability to grow and produce ATP, whereas in the absence of metal(loid)s, no growth or ATP production were observed. The protonophore carbonyl cyanide m-chlorophenylhydrazone depressed metal(loid) reduction. Anaerobic tellurate respiration will be a significant component in describing biogeochemical cycling of Te at hydrothermal vents.

5.2. INTRODUCTION

The metalloid tellurium (Te), a group 16 element related to oxygen and sulfur, possesses chemically stable oxidation states of +VI (tellurate), +IV (tellurite), 0 (elemental tellurium) and –II (telluride), with most Te occurring as tellurate in the hydrosphere and as tellurides of gold and silver in the lithosphere (*33*). The extremely low mean global abundance of Te (10^{-2} to 10^{-8} ppm) (*33*) conceals the fact that its distribution is highly heterogeneous, occurring at concentrations of 12 to 17 ppm in hydrothermal vents (*10*), 14.8 ppm in gold mines (*32*) and a surprising 30.6 ppT in mineral scales in some geothermal pipelines (*23*).

While oxidized Te is toxic to most microorganisms at concentrations as low as 1 μg/ml (*28*), aerobic reduction allows some species to resist K_2TeO_3 as high as 2500 to 5000 μg/ml (*20, 22, 33, 34*). Most reported tellurite and tellurate resistance occurs aerobically, but a few important anaerobic exceptions exist. For example, the purple bacterium *Rhodobacter sphaeroides* reduces tellurite at concentrations of up to 600 μg/ml to dispose of excess reducing equivalents generated during anaerobic photosynthesis (*14*). Resting cells of the sulfate reducer *Desulfovibrio desulfuricans* couple the reduction of tellurite to the oxidation of formate, but cannot conserve the energy from this reaction (*11*). No dissimilatory electron transport to Te compounds is known to date (*6, 33*), even though the energetics of the TeO_3^{2-}/Te redox couple (0.827 V) are more favorable for anaerobic respiration than the SO_4^{2-}/HS^- redox couple (-0.217 V) (*11*), utilized by sulfate reducers. Perhaps toxicity of tellurite, combined with its low mean global abundance has

led to the belief that Te is biologically unimportant to microorganisms. However, tellurate is less toxic to microorganisms than is tellurite (*33*). Furthermore, respiration on toxic oxidized Se, As and V is known (*12, 26*). Te, Se and V are concentrated in both hydrothermal and industrial environments (*10, 23, 32, 33*), which select for bacteria that efficiently metabolize high concentrations of oxidized metal(loid)s.

Hydrothermal vents are geological formations that release altered seawater, which has been heated, often to temperatures exceeding 400°C, by subterranean magma pockets as it circulates through the crust, mobilizing metals from basalt and acquiring elevated levels of sulfide from the reduction of sulfate by ferrous iron dissolved in the hot fluid (*30*). In the deep ocean, hydrothermal vents host unique communities of animals. Sulfide worms, tubeworms and ciliates harbor bacteria with unusual metal(loid)-associated metabolisms. Alvinellid sulfide worms, such as *Paralvinella sulfincola*, are polychaetes that graze bacterial mats on black smoker chimneys (*30*). They are exposed to high concentrations of heavy metals (*30*) and are covered with a community of metal-resistant bacteria (*8*). *Ridgeia piscesae* is a tubeworm that forms bush-like growths near black smokers. Its internal bacterial symbionts utilize sulfide as an energy source to fix carbon (*30*). The proximity of worms to anoxic vent fluids suggests that they might host anaerobic metal(loid)-respiring epibiotic bacteria. One proposed basis of this association is the detoxification of invertebrate surfaces by symbiotic microorganisms (*2, 7, 8*). This idea is supported by the observation that bacteria isolated from hydrothermal vent fauna often produce exopolysaccharides with metal binding properties (*7, 31*). Our research

indicates that specialized dissimilatory metabolic pathways employed by bacterial epibionts are another important detoxification strategy in such associations.

5.3. MATERIALS AND METHODS

5.3.1. Enumeration and Isolation. In July 2002 the submersible ROPOS was used to obtain samples for microbial culturing from two hydrothermal vent fields in the Eastern Pacific Ocean. In 2003, samples of sulfide worms (*P. sulfincola*) and tube worms (*R. piscesae*) were collected for enumeration of their external metal-resistant bacterial communities. Vent samples were acquired either by 'suction sampling', in which fine material is drawn into a sample jar through a hose by negative pressure and is then hermetically sealed during transport to the surface, or by placing larger pieces into a sealable acrylic box using the submersible's manipulator arm. Sampling sites are described in Results and Discussion. For enumeration, animals were washed with sterile dilution medium. Agar deep tubes and aerobically incubated plates were inoculated in decimal dilution series on medium PG, a modification of metavanadate respiration medium (*12*), amended with Na-lactate (1 g/l) and one of the metal(loid)s, K_2TeO_3 (300 $\mu g/ml$), K_2TeO_4 (300 $\mu g/ml$), Na_2SeO_3 (1000 $\mu g/ml$), $NaVO_3$ (900 $\mu g/ml$) or Na_3VO_4 (1000 $\mu g/ml$). Resistance to metal(loid)s was assessed by differential counts in the absence and in the presence of the above concentrations of metal(loid). Anaerobic enrichment cultures in completely filled screw-capped tubes and in crimp-sealed tubes under a headspace of N_2 were also established in medium PG at concentrations described above. Inoculum was prepared for enrichment cultures by crushing animals by hand in

aseptic plastic bags to homogenize them. Plates of medium PG were streaked from enrichments and about 100 metal(loid)-reducing strains were purified based on visual detection of the color change indicative of metal(loid) reduction that develops in colonies during incubation. Reduction of selenite, tellurite, tellurate, metavanadate and orthovanadate (all colorless in solution at the concentrations employed) is indicated by the appearance of bright red elemental Se, black elemental Te, bluish-green V^{IV} and black V^{II} (*12, 22*). For assessment of anaerobic metal-amended growth of purified strains on plates, cell suspensions were spread on the surface of agar containing medium PG amended with metal(loid)s at aforementioned concentrations and incubated in Gaspak anaerobic jars at room temperature.

5.3.2. Kinetics Experiments. Aerobically grown cells of ER-Te-48, ER-V-6 and AV-V-25 were injected into 120-ml crimp-sealed bottles containing 80 ml of PG medium amended with tellurate (0 and 300 µg/ml), metavanadate (0 or 900 µg/ml) or orthovanadate (0 or 1000 µg/ml) under a headspace of N_2. Tellurate-supplemented cultures were incubated either in the absence of added organics or in the presence of acetate (1 g/l), lactate (1 g/l), or yeast extract (0.2 g/l), singly or in combinations. Metavanadate-amended cultures were supplied with lactate (1 g/l) and yeast extract (0.2 g/l), and cultures grown with orthovanadate were supplemented with galactose (1 g/l) and yeast extract (0.2 g/l). Samples of tellurate respiring cultures were collected at intervals of several days for determination of CFU/ml as an assay of growth, supplemented with microscopic analysis to verify the absence of cellular aggregation. Growth of vanadate

respiring organisms was assessed over intervals of several hours, by total cell protein measurement using the Bradford method (*4*).

5.3.3. Protonophore Experiments. To investigate the effects of the protonophore and respiratory inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on anaerobic metal(loid) reduction and growth, anoxic metal(loid)-amended cultures were supplemented with 0, 0.2, 0.5, 1.0, 10, 20 and 50 µM CCCP (*18*) and the color intensity of reduced forms of respective metal(loid)s was assessed by measuring the values of reflectance of the red, green and blue channels using Adobe Image Ready 3.0 software applied to digital photographs of cultures (JPEG files in RGB format) obtained using an Olympus C-2020 Z digital camera. Metal(loid)s were added at levels indicated in the section on Enumeration and Isolation.

5.3.4. ATP Experiments. Anaerobic liquid cultures were established as described under "Kinetics Experiments". Metal(loid) concentrations used were 0, 10, 100 and 300 µg/ml (tellurate) or 0, 300, 1000 and 2000 µg/ml (metavanadate). Samples were collected at specified time intervals and analyzed for ATP concentration by the luciferin-luciferase bioluminescence assay (Adenosine 5'-triphosphate Bioluminescence Assay Kit, Sigma Chemical Company) following extraction of ATP from samples by perchloric acid (*25*).

5.3.5. Phylogenetic Analysis. Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene sequences and direct sequencing of the purified PCR products were carried out as described (*21*). DNA G + C content was determined by using HPLC (*13, 27*).

5.4. RESULTS AND DISCUSSION

5.4.1. Enumeration and Isolation. Enumeration of the cultivable metal(loid)-resistant aerobic and anaerobic bacteria inhabiting sulfide worms and tubeworms from the main Endeavor vent field of the Juan de Fuca Ridge ($47^o54'$ N; $129^o6'$ W) suggested that the occurrence of anaerobic metal(loid) reduction among bacterial epibionts depends on the animal species with which they are associated (Fig. 5.1). The color of the colonies in agar deep cultures indicated that bacteria were not only resistant to, but also reducing the metals to which they were exposed. Between 10 and 54% of the anaerobic epibionts of sulfide worms were resistant to selenite, tellurite and tellurate, but a smaller proportion expressed aerobic resistance, suggesting the existence of anaerobic respiration in this bacterial community (Fig. 5.1A). In contrast, metavanadate and orthovanadate resistance were dramatically greater aerobically than anaerobically, and more growth occurred in the presence of vanadates than in their absence (Fig. 5.1A). This observation suggests that sulfide worm epibionts may benefit from V aerobically, just as *Azotobacter* utilizes V in place of Mo in nitrogenases (*17*). The tubeworm epibiont enumeration revealed that anaerobic metal(loid) reduction is more prevalent in association with this animal than with sulfide worms. Anaerobically, 23 to 100% of the total tubeworm epibionts appeared in the presence of metal(loid)s, while only 23% could grow in their absence (Fig. 5.1B). The lower counts on metal-free media may be the result of the inability of the bacteria to find alternate electron acceptors for anaerobic respiration. This preferential growth on metal(loid)s is strongly suggestive that metal(loid) respiring bacteria are well-represented in the epibiotic microflora of tubeworms. Furthermore, a much smaller proportion of

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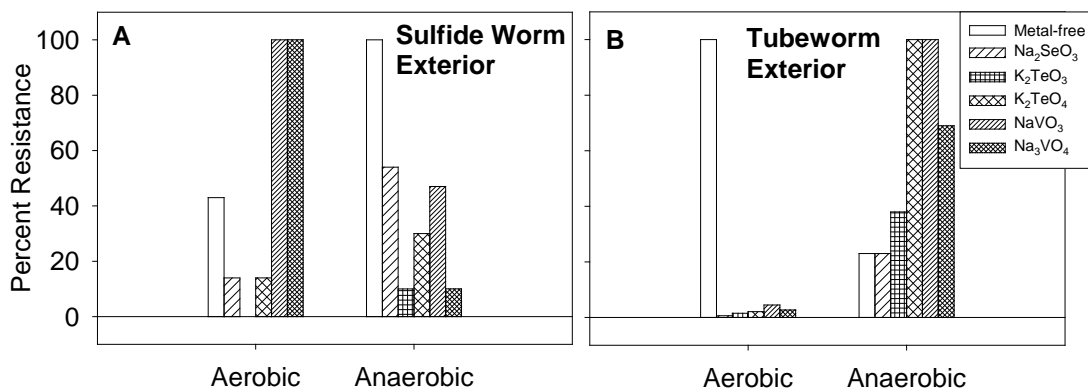
Fig. 5.1. Percent resistance of cultivable aerobic and anaerobic epibiotic bacterial communities to Na_2SeO_3 (1000 µg/ml), K_2TeO_3 (300 µg/ml), K_2TeO_4 (300 µg/ml), $NaVO_3$ (900 µg/ml) and Na_3VO_4 (1000 µg/ml). Samples: **(A)** sulfide worms and **(B)** tubeworms. MF, metal-free treatment. All populations are normalized to the highest CFU value measured for each animal and oxygenation treatment, which is assumed to represent 100% of the bacterial population under those conditions. Values provided are the mean calculated from three samples each.

aerobic organisms were resistant to any of the metal(loid)s (Fig. 5.1B). Dissolved sulfide in the vent fluids that bathe the animals might chemically remove enough oxygen from the surface of tubeworms to force attached bacteria to shift from aerobic to anaerobic respiration on metal(loid)s. Tubeworms rely on mutualistic sulfide oxidizing chemoautotrophic endosymbionts to provide them with fixed forms of carbon, requiring them to more consistently expose their branchial plumes to sulfide rich fluids than sulfide worms, which lack mutualistic endosymbionts (*30*). Hence, tubeworm integument should be a more oxygen-free environment, explaining why the anaerobic metal(loid) reducing bacterial population was more pronounced in tubeworm samples than in sulfide worm samples.

We isolated over 100 strains of facultatively anaerobic metal(loid)-resistant bacteria from metal(loid)-amended anaerobic enrichment cultures from hydrothermal vent fauna and chose four strains for detailed physiological work. Both tellurate-reducing strain ER-Te-48 and selenite-reducing strain ER-Se-17L originated from *P. sulfincola* from the Lucky Find site within the Explorer Ridge vent field (49°45'38" N; 130°15'23" W, 1791 m depth). Orthovanadate-reducing strain AV-V-25 was isolated from *P. sulfincola*, from Hell vent, within the Axial Volcano caldera (45°56'00" N; 130°00'51" W, 1543 m depth). Metavanadate-reducing strain ER-V-6 was isolated from a ciliate-rich blue-colored mat-like growth from the site called Tubeworm, within the Explorer Ridge vent field (49°45'35" N; 130°15'26" W, 1781 m depth). All four strains were motile rods (Fig. 5.2B-E).
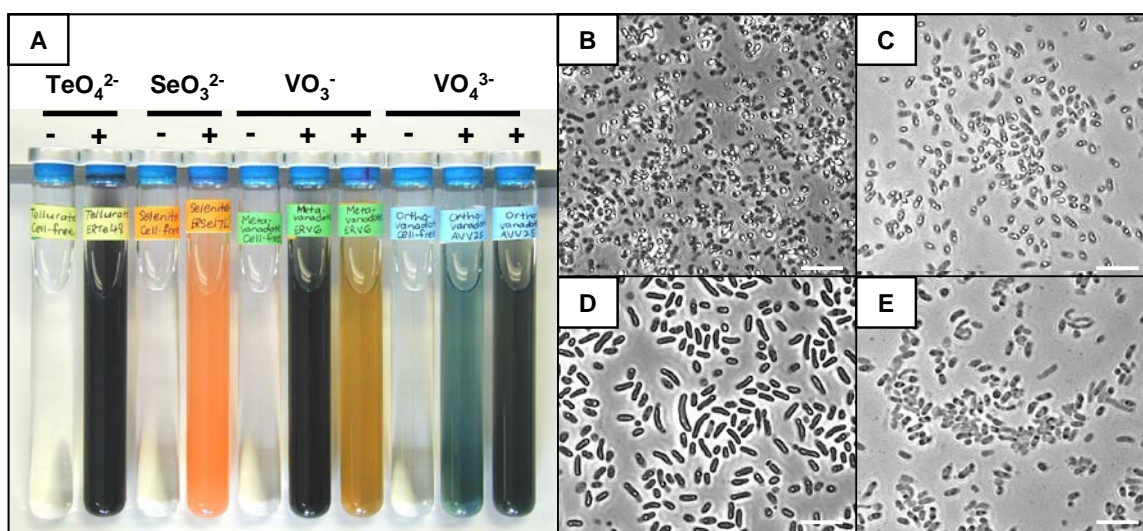
Fig. 5.2. Metal(loid) reduction and morphology of deep ocean bacteria. **(A)** Anaerobic cultures of strains respiring metal(loid)s. "-", cell-free control containing the metal(loid); "+", cultures containing the metal(loid). For metavanadate, the black and the rust-colored cultures contained 0.3 and 2 g/l organics, respectively. For orthovanadate, the blue and the black cultures contained 0.7 and 2.2 g/l organics, respectively. **(B)-(E)** Phase contrast microscopy of anaerobic metal(loid)-amended ER-Se-17L, ER-Te-48, ER-V-6 and AV-V-25, respectively.

5.4.2. Range of Metal(loid) Reduction. When cultured anaerobically in liquid medium with tellurite or tellurate, ER-Te-48 reduced a clear solution of either oxyanion to black elemental Te, accumulated intracellularly as refractile crystallites, which previous research has firmly established as Te^0 (Fig. 5.2A, C) (*14, 33*). In the presence of selenite, ER-Se-17L reduced a clear oxyanionic solution to a reddish-orange suspension of elemental Se, accumulating extracellular refractive globules of Se^0 (Fig. 5.2A, B). Neither of the V-reducers initially accumulated any precipitates when cultured on vanadates (Fig. 5.2D, E). However, ER-V-6 altered the color of the medium from a clear solution characteristic of metavanadate to black and then to rusty brown, which finally precipitated as an unidentified material (Fig. 5.2A). Formation of a black colloidal in an initially clear to yellowish solution in vanadate respiring *Pseudomonas isachenkovii* indicates the reduction of V^V to V^{II} (*12*). Anaerobic cultures of AV-V-25 turned deep bluish-green or black (Fig. 5.2A), denoting the presence of the +IV and +II oxidation states of V (*12*). Cell-free control medium did not change color in the presence of any of the metal(loid)s, confirming that abiological metalloid reduction did not occur. Furthermore, the rate of color change associated with metal(loid) reduction was faster and more intense in cell suspensions of higher density.

On aerobic agar containing medium, only the colonies of tellurate and selenite reducers accumulated persistent color. Vanadate reducing colonies initially turned the surrounding agar bluish-black, but the color was only transient, clearing after a few days. Strains also grew on anaerobic plates and in agar deeps amended with metal(loid)s, forming colonies with intense color indicative of the reduced metal(loid)s. In fact,

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reduction was more extensive anaerobically than aerobically, supporting the argument for anaerobic respiration. Under anoxic conditions, the color generated by vanadate reducers was persistent, and prolonged growth led to the development of rust colored deposits.

All strains exhibited various degrees of high level resistance to and reduction of several different metal(loid)s, tested singly (tellurite and tellurate at 300 µg/ml, selenite and orthovanadate at 1000 µg/ml, metavanadate at 900 µg/ml), both aerobically and anaerobically (Table 5.1). Aerobically, ER-Se-17L was resistant to all metalloids, but only reduced selenite strongly. Anaerobically, it was resistant to and reduced selenite and orthovanadate and, weakly, tellurate. Strain ER-Te-48 grew on all metalloids aerobically, and reduced all except the vanadates. It reduced tellurite even more effectively than tellurate. Anaerobically, it was resistant to and reduced only tellurate and tellurite, and weakly, selenite. Strain ER-V-6 grew on all metalloids aerobically and visibly reduced both Te oxyanions. Anaerobically, it survived on and reduced metavanadate and tellurate. Finally, AV-V-25, the fastest grower, survived on vanadates and selenite aerobically, but was sensitive to Te compounds and reduced only selenite. In the absence of oxygen, it was resistant to and reduced selenite and vanadates, but not tellurate.

The diversity of metal(loid)s that each strain reduced is consistent with an encounter of multiple metals by bacteria in hydrothermal vents, where resistance to just one metal(loid) would not aid in survival against the other metal(loid)s simultaneously present. Multiple resistance makes vent isolates particularly useful in bioremediation as either whole organisms or as a source of appropriate genetic elements.

Table 5.1. Resistance and reduction of metal(loid)s in vent strains.

Strain	Resistance to (and Reduction of) Metal(loid)s				
	Na_2SeO_3	K_2TeO_3	K_2TeO_4	$NaVO_3$	Na_3VO_4
Aerobic					
ER-Se-17L	+ (+)	W (W)	+ (W)	+ (-)	+ (W)
ER-Te-48	+ (+)	+ (+)	+ (W)	+ (-)	+ (-)
ER-V-6	+ (+)	+ (+)	+ (+)	+ (-)	+ (-)
AV-V-25	+ (+)	- (-)	W (W)	+ (-)	+ (-)
Anaerobic					
ER-Se-17L	+ (+)	NA	W (W)	- (-)	+ (+)
ER-Te-48	W (W)	NA	+ (+)	- (-)	- (-)
ER-V-6	- (-)	NA	+ (+)	+ (+)	- (-)
AV-V-25	+ (+)	NA	- (-)	+ (+)	+ (+)

+, good growth or reduction; W, weak growth or reduction; -, no growth or reduction; NA, not applicable.

5.4.3. Kinetics Experiments. The strongest evidence for anaerobic respiration on a particular metal(loid) is an increase in cellular biomass coupled with the reduction of the metal(loid). In anoxic liquid cultures amended with tellurate, we noted first a drop in colony count, followed by a nearly two-order of magnitude increase to 10^5 to 10^6 CFU/ml (Fig. 5.3A). This transition may involve the initial induction of enzymes in a specific metalloid respiratory pathway. Tellurate-free cultures, by contrast, dropped to and remained at 10^3 CFU/ml or less. The initial drop in CFU may indicate (1) the manifestation of toxic effects of tellurate prior to the induction of enzymes that allowed for energetic utilization of tellurate and (2) the depletion of energy reserves of cells that had no alternate metabolic strategy available prior to induction of tellurate respiration enzymes. Because cultures in the absence of tellurate continued to lack an alternate electron acceptor, they never recovered from the initial drop. Cultures with the most extensive growth also showed the highest degree of tellurate reduction, evident from the dark color of the medium. When cultured on a single carbon source, reduction and growth were most extensive in the presence of lactate. Amendment of cultures with various combinations of lactate, acetate and yeast extract indicated that lactate and yeast extract stimulated growth and reduction, with the latter probably acting as a growth factor, while acetate did not serve as a source of reducing power for bioreduction.

Anaerobic cultures of ER-V-6 and AV-V-25 showed an appreciable increase in biomass only in the presence of metavanadate and orthovanadate, respectively (Fig. 5.3B, C), distinguishing this process from electron disposal strategies that simply maintain redox poise, such as in anaerobic phototrophs (*14*). Unlike tellurate reducing ER-Te-48,
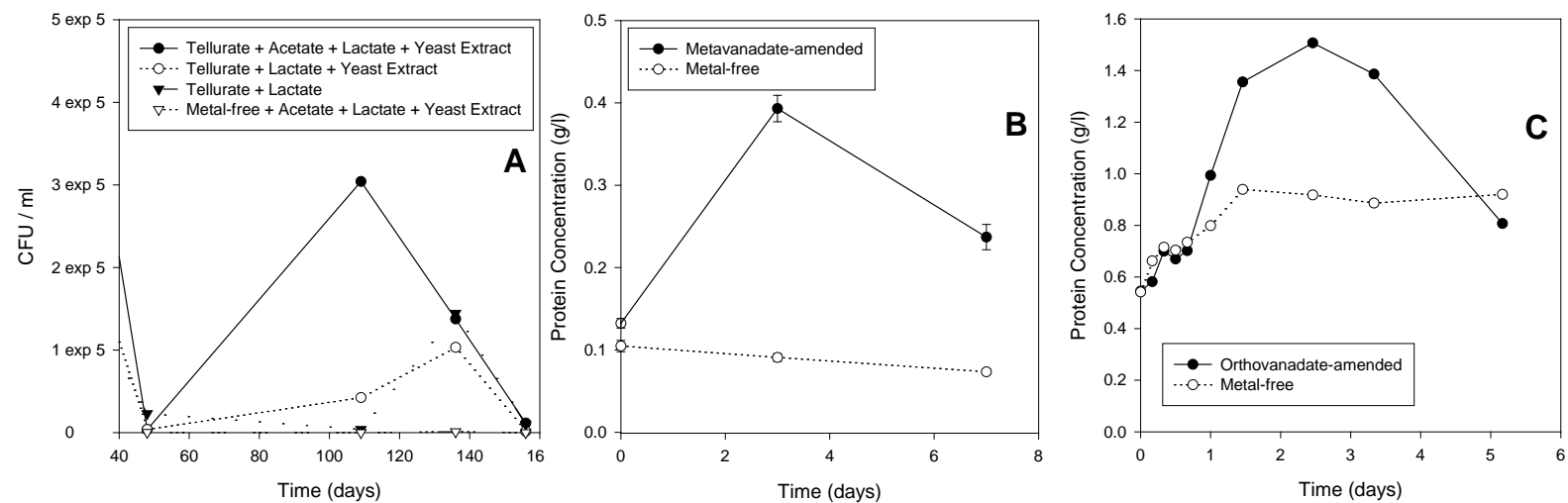
Fig. 5.3. Anaerobic respiratory growth kinetics. **(A)** ER-Te-48 with various organics, with and without tellurate. **(B)** ER-V-6 with and without metavanadate. **(C)** AV-V-25 with and without orthovanadate. Metal(loid) concentrations as described in Fig. 5.1. Error bars refer to standard error from triplicate cultures.

they showed no initial decrease in biomass, and development was much faster, reaching stationary phase within 2 to 3 days. Thus metal respiratory systems in both strains are either induced much faster than in the tellurate reducer, or they are constitutively expressed. Such a trait would be useful to bacteria utilizing vanadate for respiration at deep ocean hydrothermal vents, as levels of V are often very high in this environment (*33*). The distribution of Te on the other hand, is more variable (*33*), and constitutive expression of tellurate respiration systems may be wasteful to cells.

5.4.4. Protonophore Experiments. The inhibitory effects of protonophores on metal(loid) reduction may indicate anaerobic respiration because protonophores collapse the transmembrane pH gradient that is established during respiration to generate ATP from ADP, a reaction which in sulfate reducing bacteria is involved in a preparatory step of the reduction of sulfate to sulfite (*15, 24*). We investigated the effects of the protonophore CCCP on anaerobic reduction of tellurate, selenite, metavanadate and orthovanadate. Anaerobic metal(loid)-amended cultures supplemented with 10 and 50 μM CCCP resulted in no growth or metal(loid) reduction, while CCCP-free cultures reduced metal(loid)s. Oremland et al. (*18*) reported similar results for cultures of selenate-respiring strain SES-3. At such high concentrations, CCCP also inhibited growth aerobically. At 1.0 μM, CCCP did not arrest aerobic growth, but anaerobically it had increasingly inhibitory effects on ER-Se-17L, ER-Te-48 and ER-V-6 as CCCP concentration increased from 0 to 0.2, 0.5 and 1.0 μM. AV-V-25, the fastest growing strain, showed no difference in performance between 0 and 1.0 μM. We interpret these

results to mean that the proton gradient in anaerobically slowly growing strains is more easily quenched by the ionophore than in fast growers. Contrary to previously published short duration (a few minutes) experiments on resting cells in which an initial increase in the reduction of the terminal electron acceptor is typically observed (*1*), our long-term growth experiments (several days in duration) focused on preventing growth (and therefore metal(loid) reduction) by the only method of metabolism available for exploitation under the conditions provided. Under these conditions, growth and metal(loid) reduction in metal(loid) respiring strains should be inhibited concurrently by a protonophore.

5.4.5. ATP Experiments. Organisms that carry on anaerobic respiration generate ATP from the coupling of the oxidation of a carbon source with the reduction of a terminal electron acceptor. In our case, metal(loid)s served the purpose of this acceptor. The rate of ATP generation should increase with metal(loid) concentration to a maximum at some optimum concentration, and then decrease with further increases in metal(loid) concentration as toxic effects of the element outweigh its utility as an electron acceptor. We measured the intracellular ATP levels of cell suspensions in the presence of various metal(loid) concentrations for the tellurate and metavanadate reducers. As cells of ER-Te-48 aged, ATP declined much more quickly in the dying metal-free cultures than in cells in the presence of 10, 100 or 300 μg/ml of K_2TeO_4, which serves as a terminal electron acceptor to temporarily support metabolism in cell suspensions. At a point when dead tellurate-free cells contained very low ATP levels (only 20 nM), cultures amended

with 10 μg/ml of K_2TeO_4 possessed 50 times more ATP (1068 nM). Greatest ATP regeneration was at 10 μg/ml of K_2TeO_4 (Fig. 5.4A), comparable to Te concentrations reported for deep ocean hydrothermal vents (*10*). Cells of the metavanadate reducer ER-V-6 showed a similar trend, although ATP was regenerated most efficiently in media amended with the highest concentration of $NaVO_3$, 2000 μg/ml (Fig. 5.4B), a concentration of V sometimes encountered in the environment (*33*). The rapid ATP decline in the absence of metalloids indicates that cells could not oxidize lactate because of the lack of an appropriate terminal electron acceptor. While our experiments did not track exponentially growing cultures, addition of tellurate or metavanadate to resting cell suspensions greatly increased the levels and duration of these levels of ATP in metal(loid)-amended cultures relative to metal(loid)-free cultures, demonstrating the ability of cells to conserve energy from electron transport to metal(loid)s.

5.4.6. Phylogenetic Analysis. Partial sequencing of 16S rRNA revealed that strains ER-Se-17L, ER-Te-48 and ER-V-6 were 99.3 to 99.8% similar to the type strain of *Shewanella frigidimarina*, isolated from Antarctic ice and capable of anaerobic Fe(III) respiration (*3*). Members of *Shewanella* are among the most metabolically versatile bacteria in their diversity of metal resistance and anaerobic respiration (*16*). *Shewanella oneidensis* was recently shown to be capable of metavanadate respiration (*5*). However, ER-V-6 is the first metavanadate respiring *Shewanella* relative isolated from deep ocean hydrothermal vents. Furthermore, unlike *S. frigidimarina*, ER-V-6 was cultured from invertebrates, implying a possible symbiotic association with vent polychaetes. This
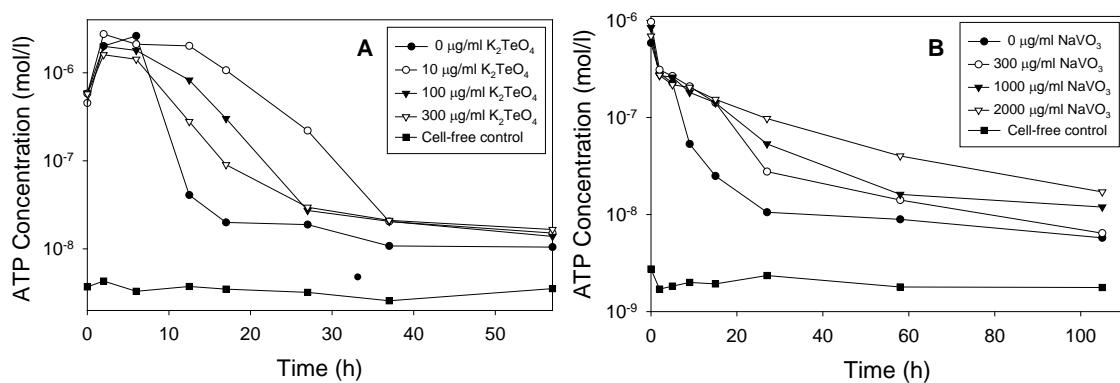
Fig. 5.4. Intracellular ATP concentrations. **(A)** ER-Te-48 with and without tellurate. **(B)** ER-V-6 with and without metavanadate.

hypothesis is theoretically sound on the basis of a detoxification/nutrient acquisition mutualism (*2, 7, 8*). Associations between metal respiring bacteria and invertebrates are known from non-vent systems (*12*). Metavanadate respiration has been reported for *Pseudomonas* species (*12*) and for *Geobacter metallireducens* (*19*), none of which come from hydrothermal vents. While respiration on selenite is known for microorganisms from hydrothermal systems, it has only been documented for the archaean *Pyrobaculum*, and only from terrestrial solfatara (*33*). Even more interesting is the demonstration of orthovanadate respiration in AV-V-25, which was 99.8% similar to *Vibrio pomeroyi*, isolated from larvae of the bivalve *Nodopecten nodosus* from southern Brazil (*29*). Bacteria of this genus are not well known for their metal resistance, much less for dissimilatory reduction of toxic metal oxyanions. Our strain is also the first known organism capable of respiring on orthovanadate rather than the usual form of pentavalent V (metavanadate) utilized in microbiological experiments. Anaerobic reduction of tellurite occurs in *Desulfovibrio desulfuricans* (*11*), *Rhodobacter sphaeroides* (*14*) and *Shewanella oneidensis* (*9*), but in none is it reported to be dissimilatory in nature, and no reduction of Te oxyanions has previously been reported for *S. frigidimarina*.

Genetic affinities were further supported by G + C contents of 42.4, 42.4, 41.0 and 43.1 mol% for ER-Se-17L, ER-Te-48, ER-V-6 and AV-V-25, respectively. However, despite the high sequence similarities, phenotypic characterization must be performed for proper identification. Detailed taxonomic characterization of isolated strains is forthcoming.

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Chapter 6.

Roseovarius vanadiphilum, **sp. nov., an extremely 'vanadiphilic'**

multiply metal-resistant and halotolerant aerobic anoxygenic

phototroph from hypersaline springs in Canada.

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The first author was the major contributor to research presented.

6.1. Abstract

Two pinkish-peach-colored strains of obligately aerobic phototrophic bacteria, EG13[T] and EG8, were isolated from a saline spring effluent stream in west central Manitoba, Canada. Strains possessed bacteriochlorophyll *a* incorporated into a typical purple bacterial light-harvesting complex 1 (870 nm) and reaction-center (801 nm). Analysis of 16S rRNA gene sequences indicated a relationship to *Roseovarius tolerans* (99.0%). However, phenotypic characteristics set them apart. The strains were physiologically well adapted to the high salinity (0-22%), fluctuating pH (7-12) and temperature (7-40°C) of the exposed hypersaline stream of East German Creek. EG8 and EG13[T] were also highly resistant to the toxic metal(loid) oxyanions tellurite, selenite and metavanadate ($\geq$1000 µg/ml each). Most intriguingly, growth and pigment production of EG13[T] on glutamate minimal medium was stimulated by 1000-10000 µg/ml of sodium metavanadate compared to metal-free conditions. Phylogenetic analysis and phenotypic properties such as pigment composition and morphology support the proposal of the novel species *Roseovarius vanadiphilum* sp. nov., with EG13[T] as the type strain.

6.2. Introduction

The aerobic anoxygenic phototrophs (AAP) are peculiar relatives of purple nonsulfur bacteria that require oxygen for the synthesis and function of their photosynthetic apparatus, are incapable of autotrophic growth (deriving energy from either chemoheterotrophy or photoheterotrophy), possess abundant carotenoid pigments, and generally only synthesize bacteriochlorophyll (BChl) in the dark (Yurkov and Csotonyi, 2009). AAP constitute a substantial proportion (often exceeding 10%) of all bacteria in several major ecosystems worldwide, and are especially well represented in extreme habitats, with over half of the described species hailing from thermal, saline or acidic environments (Yurkov and Csotonyi, 2003, 2009). They have now also been isolated from arid biological soil crusts (Csotonyi *et al.*, 2010). Recently, cultivable anoxygenic phototrophs were enumerated in a unique Canadian saline spring system exhibiting total dissolved solids ranging from 56.7‰ to 67.3‰ (Csotonyi *et al.*, 2008). From aerobic microbial mats dominated by oxygenic phototrophs, halophilic AAP were found to be the dominant anoxygenic phototrophs (Csotonyi *et al.*, 2008).

Many AAP, especially those species from extreme environments, also exhibit inordinately high levels of resistance to toxic oxyanions of metal(loid)s (Yurkov and Csotonyi, 2003). Yurkov *et al.* (1996) demonstrated MICs ranging from 1200 to 2700 µg/ml of potassium tellurite in seven species of AAP isolated from hot springs and salt flats, considerably higher than the anaerobic anoxygenic phototrophic maximum of 900 µg/ml reported for *Rhodobacter sphaeroides* (Moore and Kaplan, 1992), and two to three orders of magnitude higher than the typical MIC of <5 to 50 µg/ml for most non-

phototrophs (Yurkov and Beatty, 1998). The reason for this widespread resistance is unclear, but the accumulation of large intracellular deposits of elemental metal(loid)s in most AAP in which metal(loid) resistance has been investigated implies the presence of efficient metal(loid) reductases (Yurkov and Beatty, 1998; Yurkov and Csotonyi, 2003). Although resistance to vanadium oxyanions has only been reported in one AAP species, *Citromicrobium bathyomarinum*, this organism reduces sodium orthovanadate at a concentration of 2000 µg/ml, evidenced by the bluish-green color acquired by VO_4^{3-}-amended cultures (Yurkov and Csotonyi, 2003). In a few species of AAP (e.g. *Roseococcus thiosulfatophilus*), metal(loid)s do not elicit a strong reduction response (Yurkov *et al.*, 1996). However, in none of them, in fact, in no known anoxygenic phototroph, does a normally toxic metal(loid) stimulate the growth of the organism at high concentration. Here we report on the anoxygenic phototroph, EG13[T], that is 'vanadiphilic', or stimulated by vanadium under aerobic conditions.

6.3. Methods

6.3.1. Isolation and cultivation

East German Creek is an inland hypersaline spring system dominated by NaCl, in West Central Manitoba, Canada. Springs generate effluent streams in which anaerobic purple bacterial microbial mats are overlain by thick growths of cyanobacteria and the green alga *Percursaria percursa* (Londry *et al.*, 2005; Csotonyi *et al.*, 2008). The pinkish peach-colored aerobic strains EG8 and EG13[T] were isolated from such habitats, with total

dissolved solids content of 65.9‰ and 67.3‰, respectively, and pH values of 6.9 and 7.5, respectively (Csotonyi *et al.*, 2008). The strains were aerobically cultivated in continual darkness at 28°C on Medium A (Csotonyi *et al.*, 2008), a variant of rich organic medium for AAP (Yurkov, 2006).

6.3.2. Morphological, physiological and biochemical tests

Phase contrast microscopy, physiological tests for salinity, temperature and pH tolerance, antibiotic resistance, anaerobic growth with various electron donors, aerobic utilization of organic carbon sources, presence of lipase, gelatinase, catalase and oxidase were determined as previously described (Yurkov *et al.*, 1994; Csotonyi *et al.*, 2008). Requirement for vitamins was tested on three successive subcultures in tubes of liquid Medium A2 (basal Medium A supplemented with 1 g/l malate, pyruvate, acetate and lactate, and 2 g/l glutamate), and amended with vitamin solutions (Yurkov, 2006) from which one of the following was removed for each treatment: biotin, B$_{12}$, nicotinic acid and thiamine. Organic carbon utilization was tested using basal carbon-free Medium A, to which the following components were added singly (1 g/l): acetate, bactopeptone, butyrate, casamino acids, citrate, ethanol, formate, fructose, glucose, glutamate, glycerol, glycolate, lactate, malate, methanol, propionate, pyruvate, succinate, sucrose and yeast extract. Autotrophy was tested by measuring growth in two serial transfers of aerobically incubated liquid basal Medium A amended with 1.5 g/l NaHCO$_3$ as carbon source and 0.5 g/l Na$_2$S$_2$O$_3$ as an electron donor.

6.3.3. Pigment analysis

Presence of BChl *a* and carotenoids was determined spectrophotometrically in cultures that had been grown aerobically in liquid Medium A for 5 days. Following centrifugation, pigments were extracted from cells in acetone/methanol (7:2, v/v). Absorbance properties of pigments incorporated into the reaction center (RC) and light harvesting (LH) complexes were determined *in vivo* using a spectrophotometer (U-2010; Hitachi), as described (Yurkov *et al.*, 1994). BChl content was measured according to Yurkov and van Gemerden (1993), using λ_{max}=770 nm and an extinction coefficient of 76 $mM^{-1}cm^{-1}$ in acetone/methanol (7:2).

6.3.4. DNA G + C content and 16S rRNA gene sequence analysis

Determination of the DNA G + C content and phylogenetic analysis based on nearly complete 16S rRNA gene sequences (greater than 1400 nucleotides long) were performed as described (Csotonyi *et al.*, 2008).

6.3.5. Metal(loid) resistance

Resistance to the metal(loid)s sodium metavanadate ($NaVO_3$), sodium selenite (Na_2SeO_3) and potassium tellurite (K_2TeO_3) was assayed by visually scoring growth on plates of Medium A amended with 0, 50, 500 and 1000 μg/ml of the metal(loid) after one week of growth. High level tolerance of strain EG13[T] to $NaVO_3$ was further investigated in liquid

cultures of a minimal glutamate (1 g/l) variant of Medium A by measuring the biomass (determined by the Bradford Assay; Bradford, 1976) of 7-day-old cultures amended with metavanadate (0, 1000, 5000, 7500, 10000 µg/ml). Pigments were extracted into acetone/methanol (7:2), and the content of carotenoids (λ_{max} = 482 nm) and BChl (λ_{max} = 770 nm) were determined spectrophotometrically for cultures from each $NaVO_3$ concentration. The ability of EG13[T] to dissimilatorily reduce metavanadate was investigated in anaerobic agar deeps and completely filled tubes of liquid minimal glutamate Medium A supplemented with 1000 and 5000 µg/ml of $NaVO_3$.

6.4. Results and Discussion

6.4.1. Morphology and culture characteristics

Both strains EG13[T] and EG8 formed small, ~ 2-3 mm-diameter pinkish-peach-colored glistening convex colonies on the surface of agar media. The color was identical to this in liquid cultures of Medium A, but in Medium A2, cultures were pink in EG13[T] and orange-tinted pink in EG8, achieving this color in 48 h. At low rotory incubator speed (40 rpm), EG8 tended to flocculate. Strains EG13[T] and EG8 were rods, measuring 1.7-2.8 x 0.5 µm and 1.2-2.0 x 0.6 µm in size, respectively (Fig. 6.1). Cells typically possessed pointed thickenings at both poles, differing from the usually monopolar pointed ends of *R. tolerans*, which in that species is associated with a budding mode of reproduction (Labrenz *et al*., 1999). Chains of cells were also produced by EG13[T], in which thickenings were not only polar but also intercalary (Fig. 6.1).
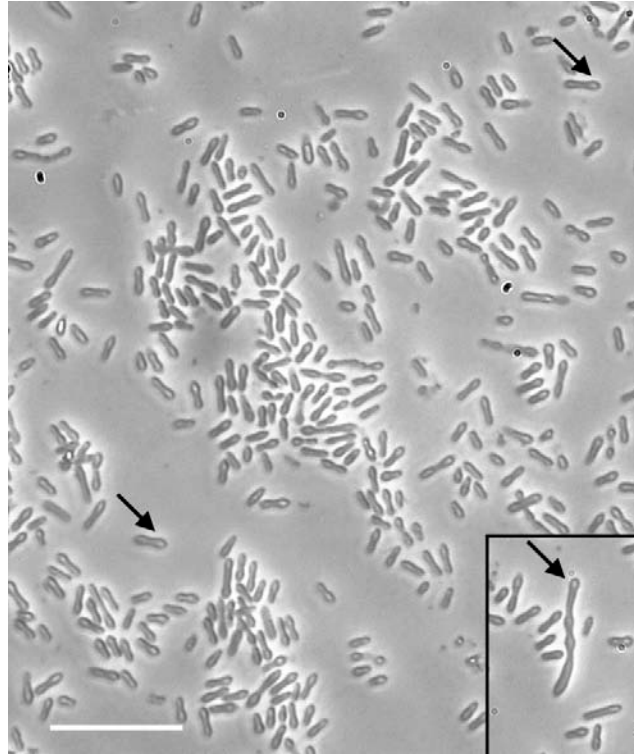
Fig. 6.1. Phase contrast micrograph of rod-shaped cells of strain EG13^T. Arrows show

polar thickenings. Scale bar: 10 μm. Inset shows chain of cells with multiple thickenings.

6.4.2. Photosynthetic characteristics

The *in vivo* absorption spectrum of EG13^T is shown in Fig. 6.2, and is similar to that of EG8. Both strains possessed BChl *a* incorporated into a RC and a single LH complex exhibiting near-IR *in vivo* absorption peaks of 801 nm (minor) and 870 nm (major) (Fig. 6.2), which is characteristic of the typical B870 purple bacterial type of LH1 (Yurkov and Csotonyi, 2009). A distinguishing feature of EG13^T and EG8 from their nearest phylogenetic neighbour, *Roseovarius tolerans* EL-172^T, is a 7 to 9 nm blue-shifting of the major LH1 peak (877-879 nm in *R. tolerans*) (Labrenz *et al*., 1999), implying a difference in the protein environment of the pigment. Absorption properties of pigment-protein complexes are generally conserved across strains within a species, and even across species within a genus, making photosynthetic pigment characteristics powerful taxonomic tools for distinguishing species and genera.

Under 500 lux incandescent illumination, EG13^T produced 46% as much BChl as it did in complete darkness. One of the nearly ubiquitous features of AAP is the complete inhibition of BChl synthesis by light (Yurkov and Beatty, 1998). The rationale is that this circumvents oxidative damage to cells caused by triplet BChl, to which light can transform BChl in the presence of oxygen (Beatty, 2002). Some species, including the related *R. mucosus*, have evolved ways to minimize the risk of exposure to triplet BChl while taking the greatest advantage of the availability of light. *R. mucosus* produces only trace amounts of BChl in the laboratory, but it up-regulates its rate of BChl synthesis on nights that follow longer days, as in summer months when environmental conditions are most conducive to growth (Biebl and Wagner-Döbler, 2006). The relative light-
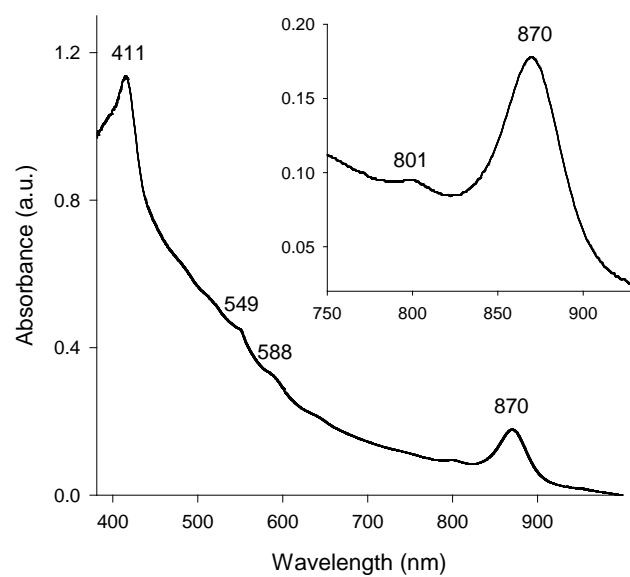
Fig. 6.2. *In vivo* absorption spectrum of pigments in EG13^T. Numerals above spectra

denote λ_{max} values. Inset highlights absorbance of BChl in LHI and RC complexes.

insensitivity of BChl synthesis in EG13[T] suggests that the organism possesses an alternative mechanism: it produces relatively abundant BChl and must therefore possess an effective defense against oxidative stress. In light of the impressively high constitutive resistance of both EG13[T] and EG8 to metal(loid) oxyanions (see below), it is perhaps not surprising to find protection against oxidative damage in these strains. Recently, some other AAP have been found to produce BChl even in the light. *Roseateles depolymerans* possessed 25% of its maximal BChl content at 10 W/m^2 light intensity (Suyama *et al*., 2002), while *D. shibae* produced BChl at 15% of its dark rate in the light (Biebl and Wagner-Döbler, 2006).

Other pigments, including carotenoids, absorbed maximally *in vivo* at 479 nm (shoulder), 511 nm (shoulder), 549 nm (shoulder) and at 642 nm in EG13[T] (Fig. 6.2). The major peak at 411 nm was due to an abundance of cytochrome *c*. When extracted with acetone/methanol (7:2), the carotenoids of EG13[T] possessed peaks at 447 nm (shoulder), 482 nm (major peak) and 505 nm (shoulder). The absorbance spectrum and color of the pigment bear similarities to bacteriorubixanthinal, which is a major component of α-4 proteobacterial AAP for which carotenoids have been enumerated, such as *Erythrobacter litoralis* (Yurkov *et al*., 1994), *Erythrobacter longus* (Takaichi *et al*., 1990) and *Erythromicrobium ramosum* (Yurkov *et al.*, 1993), but not in the α-3 proteobacterial *Roseobacter denitrificans* (Takaichi *et al*., 1991), nor in the gammaproteobacterial *Congregibacter litoralis* (Fuchs *et al*., 2007).

Growth did not occur anaerobically in either illuminated or dark conditions, and light was not required for aerobic growth. Neither acetate (1 g/l), malate (1 g/l), succinate (1 g/l), thiosulfate (0.3 g/l), nor sulfide (at 0.1, 0.36 or 0.5 g/l) served as electron donors

for anaerobic photosynthetic growth. These observations prompted the designation of EG13[T] and EG8 as AAP (Yurkov and Csotonyi, 2009).

6.4.3. Physiological traits

Both EG13[T] and EG8 exhibited very wide salt tolerance, growing with 0% to 22% NaCl (Table 6.1). This range is the highest yet published for any taxonomically described AAP. *Roseivivax halodurans*, *Roseivivax halotolerans* and *Staleya guttiformis* can grow with up to 20% NaCl by comparison (Labrenz *et al*., 2000; Suzuki *et al*., 1999), while the closest relatives of EG13[T] and EG8, the meromictic lake-dwelling *R. tolerans* and the marine *R. mucosus*, tolerate up to 10% NaCl (Labrenz *et al*., 2000; Biebl *et al*., 2005). Wide halotolerance reflects the swings in salinity expected from desiccation that alternates with meteoric dilution of stream water at EGC (McKillop *et al*., 1992; Csotonyi *et al*., 2008). Strain EG13[T] also possessed the highest known optimum salinity for growth (14%) for any investigated AAP. By contrast, EG8 grew best with only 2% NaCl. However, this large difference between optimal salt concentrations for growth is somewhat misleading, for both strains possessed a very wide range of salinity over which they produced at least 75% of their optimal yield of biomass: 2% to 18% for EG8 and 4% to 14% for EG13[T]. This disparity between the strains might stem from differences in their native microhabitats: EG13[T] originated from a much smaller stream than did EG8, and considerably farther from a source spring, so the former may have experienced a greater frequency of desiccation events.

Table 6.1. Distinguishing phenotypic traits of newly isolated strains EG13$^{\text{T}}$ and EG8 in comparison with other phototrophic members of *Roseovarius*.

Characteristic	1	2	3*	4†
Isolation source	Central Canadian brine spring runoff stream	Central Canadian brine spring runoff stream	Antarctic heliothermal, meromictic Ekho Lake	Culture of *Alexandrium ostenfeldii* (marine dinoflagellate)
Growth characteristics				
NaCl range (%)	0-22	0-22	<1.0-10.0	0.3-10.0
NaCl optimum (%)	14	2	1-8	1.0-7.0
pH range	7.0-11.0	7.0-12.0	5.3->9.0	6.5-9.1
pH optimum	7.0-8.0	7.0-8.0	6.2->9.0	6.5-8.8
Temp. range ($^{\circ}$C)	7-40	12-40	<3-43.5	15-43
Temp. optimum ($^{\circ}$C)	28	28	8.5-33.5	31
Anaerobic growth	-	-	-	-
Autotrophic growth	-	-	-	-
Catalase	+	+	+	+
Oxidase	+	+	(+)	+
Nitrate reduction	-	-	-	-
BChl *a*				
in vivo peaks (nm)	801, 870	801, 870	799-802, 877-879	Traces
Utilization of:				
Acetate	+	(+)	+	+
Butyrate	-	(+)	(+)	+
Citrate	-	-	-	-
Formate	-	-	n.d.	n.d.
Glutamate	+	+	+	+
Lactate	+	+	n.d.	+
Malate	+	+	+	+
Propionate	+	+	n.d.	n.d.
Pyruvate	+	+	+	+
Succinate	+	(+)	+	+
Fructose	-	-	n.d.	-
Glucose	-	-	-	-
Sucrose	-	-	n.d.	n.d.
Ethanol	-	-	n.d.	-
Glycerol	+	+	n.d.	+
Methanol	-	-	-	-
Bactopeptone	(+)	(+)	n.d.	n.d.
Casamino acids	+	+	n.d.	n.d.
Yeast extract	+	(+)	n.d.	+
Hydrolysis of:				
Starch	-	-	-	-
Gelatin	-	-	-	+
Tween	- (Tween 60)	- (Tween 60)	- (Tween 80)	- (Tween 80)
Antibiotic sensitivity				
Ampicillin	+	+	n.d.	n.d.
Chloramphenicol	+	+	+	n.d.
Imipenem	+	+	n.d.	n.d.

	1	2	3	4
Kanamycin	(+)	(+)	n.d.	n.d.
Nalidixic acid	-	-	n.d.	n.d.
Penicillin G	+	+	+	$\pm$
Polymyxin B	(+)	(+)	-	n.d.
Streptomycin	(+)	+	+	n.d.
Vitamin Requirement				
B$_{12}$	+	+	-[‡]	-
Biotin	+	+	-[‡]	+
Nicotinic acid	+	+	-[‡]	+
Thiamine	-	-	-[‡]	+

* Data from Labrenz *et al*. (1999).

† Data from Biebl *et al*. (2005).

‡ stimulatory, but not essential for growth.

Organisms: 1, EG13[T]; 2, EG8; 3, *Roseovarius tolerans* EL-172[T]; 4, *Roseovarius mucosus* DFL-24[T].

-, negative; (+), weak positive; +, positive; $\pm$, variable; n.d., not determined.

Optimal pH for growth was 7.0 to 8.0 (Table 6.1), reflecting the neutral (pH values of 6.9 and 7.5) source streams of EG8 and EG13[T], respectively (Csotonyi *et al*., 2008). This range was somewhat narrower than for either described phototrophic *Roseovarius* species (6.2 to ≥9.0 for *R. tolerans* and 6.5 to 9.0 for *R. mucosus*) (Labrenz *et al*., 1999; Biebl *et al*., 2005), but they grew with considerably higher maximal pH values than did at least *R. mucosus* (6.5 to 9.1; Biebl *et al*., 2005) (*R. tolerans* was tested up to pH 9.0; Labrenz *et al*., 1999). EG8 and EG13[T] tolerated pH up to 12.0 (the latter being the highest value that was tested). Tolerance of relatively high pH may reflect the alkalizing effect of abundant oxygenic photosynthesis in the algae-dominated microbial mats during the daytime (Jensen *et al*., 2010).

EG13[T] grew under a wide range of temperatures, from 7°C to 40°C, with an optimum at 28°C (Table 6.1). The lack of vegetation on the salt flats of EGC impedes neither the summer full sun nor the passage of cold air masses, requiring native microorganisms to develop capabilities to handle a large range of temperatures during the growing season. Strains EG13[T] and EG8 exhibited a range of thermal tolerance that was greater than that of *R. mucosus* (15 to 43°C) (Biebl *et al*., 2005), and nearly as great as their phylogenetically closest relative, the Antarctic meromictic Ekho lake dwelling *R. tolerans* (<3 to 43.5^{o}C) (Labrenz *et al*., 1999), but for somewhat different reasons. In the case of *R. tolerans*, thermal fluctuation derives not from an exposed habitat lacking in insulation, but from heat concentration within a heliothermal saline water body.

Heterotrophic growth occurred with a number of carbon sources, indicating flexible nutritional requirements (Table 6.1). Both EG8 and EG13[T] grew with acetate, glutamate, lactate, malate, propionate, pyruvate, succinate, glycerol, bactopeptone,

casamino acids and yeast extract. There was general agreement over substrate utilization between our strains and the published phototrophic members of the genus, *R. tolerans* and *R. mucosus*, except for butyrate, which was not used by EG13[T] for growth. No fermentation of glucose, sucrose or fructose was observed, as for *R. tolerans* and *R. mucosus* (Labrenz *et al*., 1999¸Biebl *et al*., 2005). The strains could not grow with inorganic carbon alone. This finding further supported their designation as AAP, none of which possess the capacity for autotrophy.

Strains EG13[T] and EG8 produced catalase and oxidase, but did not hydrolyze starch, Tween 60 or gelatin, and did not possess nitrate reductase. In these phenotypic characteristics, EG13[T] and EG8 resembled their nearest phylogenetic neighbours (Table 6.1). They were resistant to nalidixic acid (30 μg), were weakly resistant to kanamycin (30 μg), polymyxin B (300 IU) and streptomycin (10 μg), but were sensitive to ampicillin (2 μg), chloramphenicol (30 μg), imipenem (10 μg) and penicillin G (10 IU). These trends are similar to those of other photosynthetic *Roseovarius* species, except for polymyxin B, to which *R. tolerans* is resistant (Labrenz *et al*., 1999). Biotin, vitamin B_{12} and nicotinic acid were required for growth on defined medium, but the strains could be subcultured repeatedly in the absence of thiamine. AAP differ widely in their requirement for vitamins. Some species need to be supplied with one or more vitamins (e.g. *Porphyrobacter meromictius*, of which all strains are incapable of producing their own B_{12} and some strains require biotin; Rathgeber *et al.* 2007). However, many AAP, such as *Roseisalinus antarcticus* (Labrenz *et al.* 2005), synthesize all of their own vitamins, or are at best only stimulated by added vitamins (e.g. *Roseovarius tolerans*, stimulated by thiamine, nicotinic acid, biotin, and B_{12}; Labrenz *et al.* 1999). Strains EG13[T] and EG8 are

unusual in requiring three of four tested vitamins. This characteristic distinguished them from their closest relative, *R. tolerans* (Table 6.1). *R. mucosus* also has stringent vitamin requirements (synthesizing only one of the four vitamins listed in Table 6.1), but it differs from EG13[T] and EG8 in supplying itself with vitamin B_{12} rather than thiamine (Table 6.1). Dependence on vitamins may reflect the close association of both our strains (originating from an algal-rich mat) and *R. mucosus* (isolated from a dinoflagellate culture) with autotrophs that provided them with a reliable supply of growth factors.

6.4.4. Metal(loid) resistance

All species of AAP that have been investigated exhibit resistance to toxic oxyanions of certain heavy metals or metalloids – collectively metal(loid)s (Yurkov and Csotonyi, 2003). At present, however, only a fraction of the 56 described species of AAP have been assayed for metal(loid) resistance (and the phylogenetic affiliates of EG13[T] and EG8, *R. tolerans* and *R. mucosus*, are not among the investigated group). *Hoeflea phototrophica* is resistant to 50 to 1000 µg/ml of potassium tellurite (Biebl *et al*., 2006), but seven species in the genera *Erythromicrobium*, *Erythrobacter*, *Erythromonas*, *Sandaracinobacter* and *Roseococcus* possess even higher levels of resistance to tellurite (up to 2700 µg/ml) (Yurkov *et al*., 1996).

Despite the absence of any detectable Se, Te or V in water samples from EGC, strains EG13[T] and EG8 expressed resistance to Na_2SeO_3 (1000 µg/ml), $NaVO_3$ (2000 µg/ml) and K_2TeO_3 (1000 µg/ml). Most resistance to metal(loid)s in AAP is associated with reduction of the oxyanionic metal(loid) to its elemental form, conferring on cultures

the characteristic color of the reduced metal(loid): black for Te and red for Se. In *C. bathyomarinum*, amended with 2000 µg/ml Na_3VO_4, partially reduced V was evidenced by a bluish-green color (Yurkov and Csotonyi, 2003). On plate cultures of EG13[T] containing 2000 µg/ml $NaVO_3$, cell cultures appeared thicker and more intensely pink-pigmented, colors typical of carotenoids rather than the bluish-green of reduced V.

On minimal glutamate medium, EG13[T] was resistant to the highest concentration of $NaVO_3$ tested (10000 µg/ml, or 82 mM). It also produced three times as much biomass at this high metal concentration than in the absence of metavanadate, and 4.5 times the metal-free biomass with 7500 µg/ml of $NaVO_3$ (Fig. 6.3a). Furthermore, biomass-specific carotenoid production was doubled at 7500 µg/ml $NaVO_3$ compared to metal-free cultures (Fig. 6.3b), and BChl reached a maximal concentration (twice its metal-free levels) at 5000 µg/ml $NaVO_3$ (Fig. 6.3c). In some AAP species, presence of a large pool of photosynthetically decoupled antioxidant carotenoids, such as erythroxanthin sulfate (Yurkov *et al*., 1993), may implicate pigments in a protective role against metal(loid)-induced oxidative stress, providing a possible explanation for increased pigment production in response to metal(loid)s. However, the increase in biomass yield over a metal-free state suggests otherwise for EG13[T]. If detoxification were the reason for elevation of pigment content, then biomass yield in the presence of metavanadate should at best be equal to that in its absence. The reason for this large stimulatory effect of metavanadate is presently unclear. At no concentration did the strain reduce metavanadate, for spectrophotometry confirmed that changes in absorbance were restricted to elevated carotenoids and BChl. Therefore the organism did not irreversibly employ the oxyanion for disposal of excess reducing equivalents generated during active
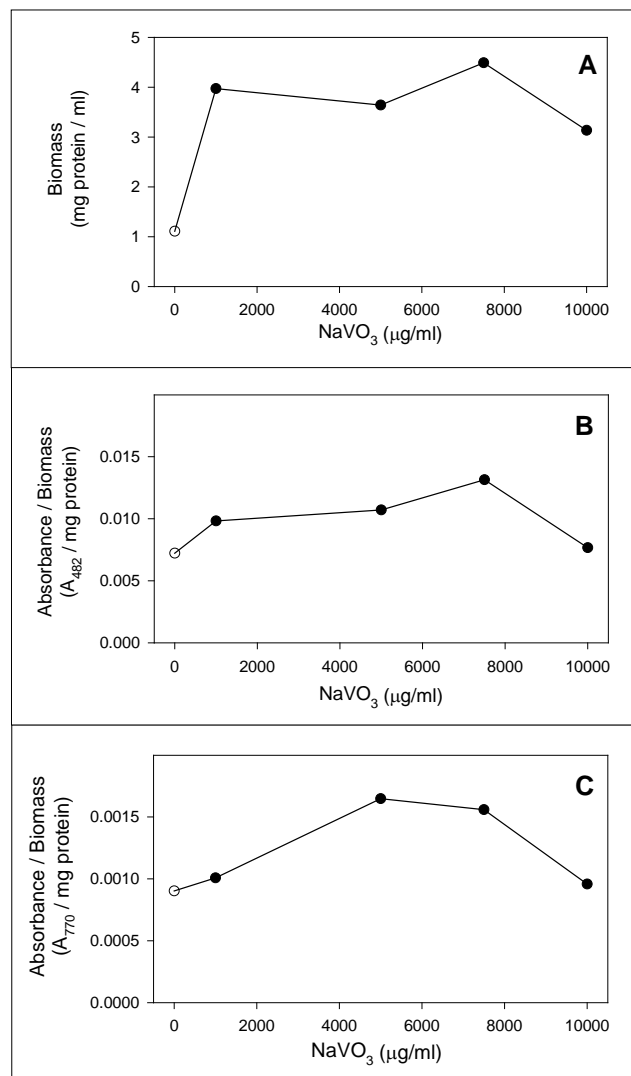
Fig. 6.3. Stimulatory effects of $NaVO_3$ on EG13[T]. **(A)** Biomass produced after 7 days of dark growth on glutamate, at $NaVO_3$ concentrations from 0 to 10000 μg/ml. **(B)** Biomass-specific absorbance of carotenoids ($\lambda_{max} = 482$ nm); **(C)** Biomass-specific absorbance of BChl ($\lambda_{max} = 770$ nm).

growth, which some anaerobic anoxygenic phototrophs appear to do with tellurite (Moore and Kaplan, 1994; Yurkov et al., 1996). Certain nitrate reducers such as *Azotobacter* can replace Mo with V in their nitrogenases (Dilworth et al., 1988), but EG13[T] was supplemented with nitrogen sources, making this role of metavanadate unlikely. This species may possess other vanadium-dependent enzymes, perhaps used in metabolizing glutamate. Alternatively, some non-phototrophic microorganisms utilize vanadate as a terminal electron acceptor in anaerobic respiration (Lyalikova and Yurkova, 1992). The AAP *Roseobacter denitrificans* is similarly capable of anaerobic respiration by utilizing nitrate as a terminal electron acceptor (Shiba, 1991). No anaerobic growth or dissimilatory reduction of metavanadate was observed in EG13[T]. A stimulatory effect of vanadate on biomass production and pigmentation was reported by Meisch and Becker (1981) in the green algae *Chlorella fusca*, in which V is suggested to act as a redox catalyst in the electron transport from photosystem II to photosystem I. However, anoxygenic phototrophs have a completely different light harvesting apparatus from oxygenic phototrophs, lacking the two-photosystem arrangement. Furthermore, the stimulatory effect of vanadate on *C. fusca* occurs in the micromolar, not millimolar range, and biomass is enhanced only 1.5-fold. Therefore, although a catalytic electron shuttling function of V is a viable explanation that will require further investigation, its nature will clearly differ substantially from that in *C. fusca*.

Why EG13[T] and EG8 are so highly metal(loid)-resistant is unclear, for spring water yielded no detectable Se, Te or V. However, because the source water appears to pass through a large volume of the lithosphere before exiting at the surface (Grasby, 2000), it may be that subterranean water conduits change course over time. This may lead

to periodic rises in metal(loid) content if the water intercepts a metal(loid) ore-rich deposit.

6.4.5. Phylogenetic analysis and DNA composition

Strains EG13[T] and EG8 were members of the *Alphaproteobacteria* and they had sequence similarity of 100%. The sequence similarity of EG13[T] to its nearest phylogenetic neighbours, *R. tolerans* and *R. mucosus*, was 99.0% and 96.6% (Fig. 6.4). The latter degree of sequence divergence was similar to what is often found between different species. Although the phylogenetic distance to *R. tolerans* was relatively low, phenotypic characteristics displayed marked dissimilarity. The DNA G+C content of EG13[T] was 64.1 mol%, similar to both the 62-64 mol% of *R. tolerans* and the 63 mol% of *R. mucosus*.

The presence of BChl *a* incorporated into RC and LH1 complexes, the inability to grow photoheterotrophically under anaerobic conditions and the absence of autotrophy prompt the conclusion that strain EG13[T] is indeed a member of the AAP, despite the relative insensitivity of BChl synthesis to illumination. Phylogenetic analysis demonstrated the proximity of EG13[T] and EG8 to photosynthetic members of the genus *Roseovarius*, *R. tolerans* (99.0%) and (more distantly) *R. mucosus* (96.6%), but morphological, physiological, biochemical and habitat properties allowed sufficient distinction. The cells showed bipolar thickenings and pointed ends, unlike the usual monopolar pointed ends of *R. tolerans*. There was a 7 to 9 nm difference in the absorption of LH1 between the EGC
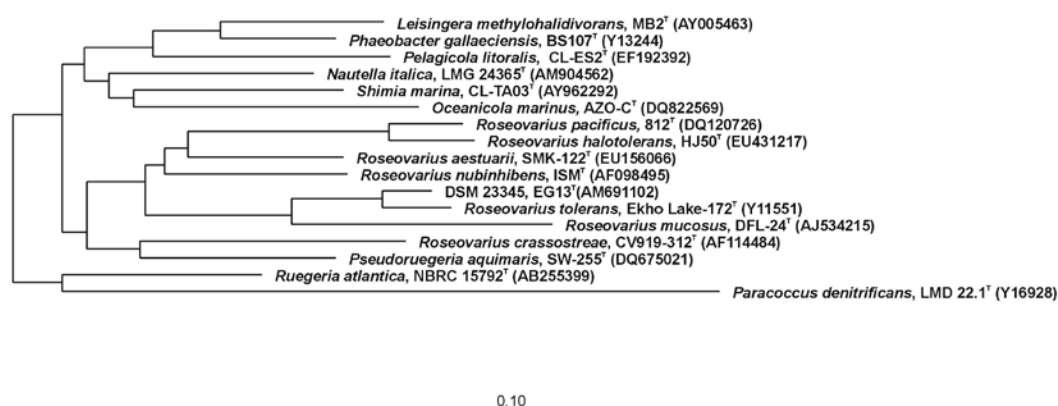
Fig. 6.4. Neighbour-joining dendrogram of 16S rRNA gene sequence relatedness, showing the position of *Roseovarius vanadiphilum* strain EG13^T and its phylogenetic neighbours, including *Roseovarius tolerans* and *Roseovarius mucosus*. The tree was rooted with the 16S rRNA gene sequences of other members of the *Alphaproteobacteria*. Bar, 10 substitutions per 100 sequence positions.

strains and *R. tolerans*, and both strains produced much more BChl than does *R. mucosus*; halotolerance exceeded that of both *Roseovarius* species; unlike *R. mucosus*, the brine spring strains synthesized vitamin B_{12} and did not hydrolyze gelatin, but unlike either *Roseovarius* species, EG13[T] and EG8 required thiamine. EG13[T] and EG8 originated from hypersaline runoff streams in a terrestrial brine spring system, unlike the marine *R. mucosus* and meromictic lake-dwelling *R. tolerans*.

On the basis of these phylogenetic and taxonomic characteristics, we propose the novel species *Roseovarius vanadiphilum*.

6.5. Description of *Roseovarius vanadiphilum* sp. nov.

Roseovarius vanadiphilum (va.na.di.phi′lum. ONorse. f. n. *Vanadis* the Norse goddess Freya, and the element vanadium; Gr. neut. adj. *philon* friend, loving; N. L. neut. adj. *vanadiphilum* vanadium-loving).

Cells are rods (1.2-2.8 x 0.5-0.6 μm). Cultures are pinkish-peach-colored due to the production of carotenoids and BChl *a*. Produce RC and LH1 complexes, with absorption peaks due to BChl at 801 and 870 nm. *In vivo* absorption peaks expressed at 411 nm, 479 nm (shoulder), 511 nm (shoulder), 549 nm (shoulder) and 642 nm. Absorption peaks in acetone/methanol (7:2) extracts at 447 nm (shoulder), 482 nm, and 505 nm (shoulder) may be attributed to carotenoid(s) such as rubixanthanal. No growth occurs anaerobically in the light, or anaerobically in the dark with sodium metavanadate as terminal electron acceptor. Obligately aerobic and facultatively photoheterotrophic. Incapable of autotrophy. No fermentation observed. Best substrates for growth are

glycerol, glutamate, propionate, malate, and lactate; growth also occurs on acetate, butyrate, pyruvate, succinate, bactopeptone, casamino acids and yeast extract. Does not utilize citrate, formate, glycolate, fructose, glucose, sucrose, ethanol, or methanol. With glutamate as carbon source, sodium metavanadate concentrations between 1000 and 10000 μg/ml stimulate growth and pigment production. Does not hydrolyse starch, Tween 60 or gelatin. Catalase positive. Oxidase positive. Nitrate is not reduced to nitrite or nitrogen. Optimum temperature for growth is $28^{\circ}C$, with growth occurring at temperatures as low as $7^{\circ}C$ and as high as $40^{\circ}C$. Optimal salinity for growth is 14% NaCl, with growth occurring over a wide range of NaCl concentrations, from 0% to 22%. Optimal pH for growth is 7.0 to 8.0, with growth occurring over a wide range of pH values, from pH 7.0 to 12.0. Resistant to the antibiotic nalidixic acid. Sensitive to ampicillin, chloramphenicol, imipenem, penicillin G, polymyxin B and streptomycin. Requires biotin, vitamin B_{12} and nicotinic acid for growth, but not dependent upon added thiamine. Member of the *Alpha-3-proteobacteria*. The DNA G + C content is 64.1 mol%.

The habitat of the first two isolated strains is a hypersaline runoff stream (total dissolved solids 65.9‰ to 67.3‰; pH 6.9 to 7.5) from the thalassohaline brine spring system known as East German Creek, near Swan River, Manitoba, Canada. The type strain is EG13[T].

6.6. Acknowledgements

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Chapter 7.

Influence of tellurite on synthesis of the photosynthetic apparatus

and pigments in aerobic anoxgenic phototrophic bacteria

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Photosynthesis Research

(To be submitted)

The first author was the major contributor to research presented.

7.1. Abstract

Despite the growing interest in bacterial-tellurite interactions, almost nothing is known about the influence of this toxic oxyanion on aerobic anoxygenic phototrophs (AAP), which possess very high level resistance to metals and metalloids. This study is the first to address the impact of tellurite on the pigments of anoxygenic phototrophic bacteria, specifically AAP. Three important effects of the metalloid on the AAP photosynthetic apparatus are reported. First, tellurite (100 μg/ml) induced enhanced expression of carotenoids and/or bacteriochlorophyll (BChl) in *Erythrobacter litoralis*, T4, *Erythromonas ursincola*, KR-99, *Citromicrobium bathyomarinum*, JF-1 and *Erythrobacter* relative, EG15. Second, the influence of tellurite on cellular pigment content depended strongly on culture conditions, particularly tellurite concentration (0–500 μg/ml) and nutrient availability (0.6–3 g/l). A five-fold decrease in organic carbon changed the effect of the metalloid from inhibitory (42%) to stimulatory (180%) on pigment synthesis in *Erythromicrobium ramosum* E5. Third, thin layer chromatography revealed that tellurite (500 μg/ml) induced the expression of BChl precursors such as Mg protoporphyrin in *E. litoralis* T4, constituting the first report of such tetrapyrole intermediates in AAP. The metalloid also increased the synthesis of some carotenoids (components 9, 12-14) in T4 but inhibited others (components 10, 11), and altered their absorption characteristics by red- and blue-shifting the peak absorption wavelengths. The results are discussed in the context of oxidative defense mechanisms induced in AAP by the presence of tellurite.

7.2. Introduction

Recent work has demonstrated that representatives of a physiological group called the aerobic anoxygenic phototrophs (AAP) possess exceptionally high resistance to oxyanions such as tellurite (Yurkov *et al.*, 1996; Biebl *et al.*, 2006). AAP are *Alpha-*, *Beta-*and *Gammaproteobacteria* discovered about 30 years ago, which can only synthesize and utilize photosynthetic light harvesting systems in the presence of oxygen (Yurkov and Beatty, 1998a; Rathgeber *et al.*, 2004; Yurkov and Csotonyi, 2009). They are incapable of anaerobic photosynthesis and photoautotrophy, distinguishing them from all other anoxygenic photosynthetic bacteria. Several AAP species were found to tolerate 2300 to 2700 μg/ml of K_2TeO_3 (Yurkov *et al.*, 1996). This is three to thirteen times as great as in anaerobic purple phototrophs (*Rhodobacter* and *Rhodopseudomonas*) which also display high level K_2TeO_3 resistance of 200 to 900 μg/ml (Moore and Kaplan 1992).

Initial work on metal(loid) resistance in AAP reported the dependence of minimum inhibitory concentration (MIC) on organic substrate type in the species *Erythrobacter litoralis*, *Erythromicrobium ezovicum*, *Erythromicrobium hydrolyticum*, *Erythromicrobium ramosum*, *Erythromonas ursincola*, *Roseococcus thiosulfatophilus* and *Sandaracinobacter sibiricus* (Yurkov *et al.*, 1996). Eliminating yeast extract in the presence of single carbon sources, such as acetate, dramatically increased the tellurite resistance of these species, especially *E. ramosum*. The MIC of this organism increased from 750 μg/ml on rich organic medium to 2300 μg/ml on minimal acetate medium, whereas for *E. ursincola*, MIC rose from 1000 μg/ml to 2700 μg/ml (Yurkov *et al.*, 1996). Beyond these results, however, research has not proceeded on metal(loid)

resistance in AAP, including the effect of metal(loid)s on pigment systems. The subject

has implications for the efficiency of metal(loid) metabolism, because altered expression

of the light harvesting pigments bacteriochlorophyll (BChl) *a* or carotenoids may

influence energetics. Since several carotenoids that AAP possess in abundance exhibit

antioxidant properties, this research may also elucidate pigment-based defense

mechanisms. Although carotenoids are postulated to ameliorate photodynamic toxicity in

AAP (Beatty, 2002), their role in metal resistance has never been addressed. Although

phototrophic *Proteobacteria* are increasingly studied from a tellurite-resistance

standpoint, most research focuses on modes of uptake (e.g. Borghese and Zannoni, 2010)

or impacts on growth (e.g. Bebien *et al*., 2001; Borghese *et al*., 2004). The influence of

Te on pigments of anoxygenic phototrophs has never been reported.

7.3. Materials and Methods

7.3.1. Cultivation

Cultures of *Erythromicrobium ramosum*, E5 and *Erythromonas ursincola*, KR-99 were

cultured aerobically at 28°C in the dark on rich organic (RO) medium (Yurkov, 2006).

For *Citromicrobium bathyomarinum*, JF-1, *Erythrobacter litoralis*, T4 and its close

phylogenetic relative, strain EG15 (Csotonyi *et al*., 2008), RO medium was amended

with 20 g/l NaCl (Yurkov *et al*., 1994a), and Medium A (Csotonyi *et al*., 2008) based on

RO medium and amended with 40 g/l NaCl, was used for EG7, EG8, EG13 and EG29.

7.3.2. Overview of AAP responses to metal(loid)s

Liquid cultures of strains E5, KR-99, JF-1, T4 and EG15, both metal-free and amended with potassium tellurite (K_2TeO_3) at 100 μg/ml, were grown for 2 days to determine the influence of K_2TeO_3 on pigment expression. From 3 ml of each culture, pigments were extracted into 1 ml acetone/methanol (7:2) and analyzed using a Hitachi U-2010 spectrophotometer. This solvent was used for extraction in all experiments unless otherwise noted. Dry mass was determined from the pellet, and protein was measured by the Bradford Assay (Bradford, 1976). BChl *a* was assayed at 770 nm (in all experiments), and carotenoids were measured at species-dependent λ_{max} values between 400 and 550 nm (Yurkov and van Gemerden, 1993a).

For a similar experiment, brine spring strains EG7, EG8, EG13 and EG29 (Csotonyi *et al*., 2008) were cultured on agarized Medium A amended with 0 and 50 μg/ml K_2TeO_3; 0 and 1000 μg/ml sodium selenite (Na_2SeO_3); and 0, 50, 500 and 1000 μg/ml sodium metavanadate ($NaVO_3$). Pigments were extracted from cells scraped off plate surfaces.

7.3.3. Influence of nutrients and K_2TeO_3 concentration on pigments of E5

E5 was cultured on RO or in a low nutrient variant medium containing (g/l) only the organics acetate (0.5) and yeast extract (0.1). After 5 days in liquid media with 0, 50, 200 and 500 μg/ml K_2TeO_3, pigments were extracted into organic solvent from 10 ml of

centrifuged culture. One ml of culture from each treatment was sonicated for *in vivo* assay of LHI (λ_{max} = 870 nm) and LHII (λ_{max} = 798, 832 nm) complexes.

7.3.4. Kinetics experiment

Biomass, K_2TeO_3 reduction and pigments were measured in 1 ml samples after 0, 4, 8, 12, 17, 24, 30, 36, 48 and 72 hours in liquid RO medium cultures of T4, metalloid-free and amended with 200 μg/ml K_2TeO_3. BChl *a*, carotenoids (A_{479}) and the dominant porphyrin intermediate (A_{416}) were assayed. Tellurite reduction was determined by recording the change in reflectance of culture photographs relative to a metal-free control, after photographs were tone-balanced to a standard neutral grey card. Precise measurement of the concentration of Te was not crucial for this experiment, but this method served to document how deposits of elemental Te darkened the medium as they accumulated.

7.3.5. Thin layer chromatography

Preparation of pigments and thin layer chromatography (TLC) were performed according to the methods of Yurkov *et al.*, (1994a), using a solvent mixture of petroleum ether/diethyl ether/acetone (40:10:10). Pigments scraped from silica plates were analyzed spectrophotometrically after resuspension in acetone/methanol (7:2). The absorbance spectrum of pigment component 3 was compared to a Mg-protoporphyrin IX (MgP) standard (Frontier Scientific), which possessed peaks at 416, 552 and 589 nm.

7.4. Results and Discussion

7.4.1. Overview of AAP: Effect of tellurite on BChl and carotenoid content

Pigment contents of five closely related (α-4 *Proteobacteria*) strains: JF-1, T4, E5, KR-99 and EG15 responded in different ways to a K_2TeO_3 concentration (100 μg/ml) well below the MIC (Yurkov *et al*., 1996). *E. ramosum*, E5 displayed a somewhat similar BChl response to K_2TeO_3 as did *R. sphaeroides* to Ni^{2+} and Co^{2+}, in that BChl diminished (a factor of 0.4 times that of K_2TeO_3-free conditions) (Table 7.1). This may represent a manifestation of oxidative stress, for K_2TeO_3 is known to trigger an increase in the generation of reactive oxygen species (Zannoni *et al*., 2008). An iron-limitation response similar to that caused by Ni^{2+} and Co^{2+} in *R. sphaeroides* (Giotta *et al*., 2006) is a less likely explanation for decreased BChl expression in E5 because of the absence of strongly absorbing porphyrin Soret bands that indicate accumulation of BChl intermediates due to displacement of Fe by a competing element in key enzymes of the BChl biosynthesis pathway (Lascelles, 1966; Spiller *et al*., 1982; Csatorday *et al*., 1984; Giotta *et al*., 2006). Interestingly, strains KR-99, T4, EG15 and JF-1 all produced slightly or substantially more BChl in the presence of K_2TeO_3 than in its absence (factors of 1.10, 1.16, 1.37 and 2.15, respectively) (Table 7.1). Because the effect of K_2TeO_3 on pigment systems of anoxygenic phototrophs has never been studied, it is unknown how commonly this stimulatory effect manifests in anoxygenic phototrophs. The reason for amplified

Table 7.1. Absorption spectrum characteristics of AAP growing in presence and absence of 100 μg/ml of K_2TeO_3.

Strain	Pigment Type [1]	Concentration (nmol/mg protein)		Peak Position (nm)		Peak Shift due to Tellurite (nm) [2]
		-Te	+Te	-Te	+Te	
E5	BChl	0.4	0.2	770	770	0
	Carot.	8.1	5.4	475	477	2
	Carot.			461	457	-4
KR-99	BChl	0.9	1	770	770	0
	Carot.	15.8	18.8	479	479	0
	Carot.			452	452	0
JF-1	BChl	0.4	0.9	770	770	0
	Carot.	7.1	4.1	479	477	-2
	Carot.			452	451	-1
T4	BChl	1.3	1.5	770	770	0
	Carot.	8.1	10.8	478	478	0
	Carot.			458	453	-5
	MgP				417	
	MgP				587	
EG15	BChl	1.3	1.8	770	770	0
	Carot.	10.8	20.8	478	477	-1
	Carot.			460	455	-5
	MgP				417	
	MgP				582	

[1] Carot., carotenoid.
[2] Negative peak shift, blue-shift; positive peak shift, red-shift; abs, absorbance.

BChl synthesis is unknown, but its occurrence in intrinsically highly K_2TeO_3-resistant AAP may not be coincidental, and it may be part of a pigment-based defense strategy.

AAP produce a rich suite of carotenoids, with any single species possessing from one to over 20 different types (Takaichi *et al*., 1990; 1991; Yurkov *et al*., 1994b; Fuchs *et al*., 2007). Relative carotenoid contents reported in this experiment pertain to maximal cumulative absorbance by all carotenoids. Unlike BChl, carotenoids responded in two ways to tellurite: a change in concentration and a shift in absorbance peak (λ_{max}).

Tellurite reduced carotenoid expression in E5 to 66% of its tellurite-free content, a proportionally smaller decline than for BChl. Thus, carotenoid synthesis was less inhibited than BChl (Table 7.1). JF-1 also exhibited reduced carotenoid production (by a factor of 0.55), opposing its increase in BChl. If photosynthetic pigments are enlisted in metalloid resistance mechanisms, then BChl appears to be favoured over carotenoids; a drop in carotenoids implies deleterious effects. By contrast, the carotenoids of KR-99, T4 and EG15 all increased in the presence of K_2TeO_3, by factors of 1.20, 1.33 and 1.93, respectively (Table 7.1). Furthermore, carotenoids were stimulated by tellurite more than was BChl, indicating a possible role in oxidative defense. Carotenoids with more than 9 conjugated double bonds have especially potent antioxidant activities, including β-carotene, lycopene, zeaxanthin and erythroxanthin sulfate (Britton, 2008; El-Agamey and McGarvey, 2008). Because investigated strains possess these carotenoids in excess (Noguchi et al., 1992; Yurkov *et al*., 1993a), it is reasonable to expect such a role for at least some species.

In most strains, λ_{max} of one or more carotenoid peaks were either red- or blue-shifted under K_2TeO_3 exposure by up to 5 nm compared to Te-free cultures (Table 7.1).

In E5, the 475-nm peak experienced a 2 nm red-shift, whereas its 461-nm peak was blue-shifted by 4 nm. In only one strain, KR-99, tellurite induced no shift in the λ_{max} of carotenoids. Because λ_{max} is strongly dependent upon molecular structure, these peak shifts likely imply an altered molecular architecture. It may reflect aggregation of carotenoids (Köhn *et al*., 2008), reactions with K_2TeO_3 or reactive oxygen species (El-Agamey and McGarvey, 2008), or synthesis of specific carotenoids to deal with K_2TeO_3.

Of the AAP investigated, two organisms (E5 and T4) were selected for more detailed study of the influence of K_2TeO_3. The results shed light both on the influence of nutrient and tellurite concentration, and on the effect of tellurite on the concentration and blue or red peak shifts of individual pigments.

7.4.2. *E. ramosum*, E5: Effect of tellurite concentration and nutrient level

Strain E5, from a Russian freshwater terrestrial hydrothermal system (Yurkov *et al*., 1994a), has a well-characterized carotenoid suite (Yurkov *et al*., 1993a; 1994b) and possesses two LH complexes, permitting a more in-depth investigation of how K_2TeO_3 affects the distribution of BChl within the cell. The response was much more elaborate than the previous experiment had suggested, depending on concentrations of both K_2TeO_3 and nutrients.

7.4.2.1. Tellurite concentration

Exposure of E5 to a range of K_2TeO_3 concentrations (0 to 500 µg/ml) confirmed that K_2TeO_3 has a deleterious effect on cellular carotenoids under eutrophic conditions. Carotenoids decreased with increasing K_2TeO_3 concentration, from 62 to 26 nmol/mg dry weight (Fig. 7.1a). Simultaneously, the wavelength separation between the two chief carotenoid absorbance peaks widened from 12 nm in Te-free medium to a maximum of 20 nm with 50 to 200 µg/ml K_2TeO_3 (Fig. 7.1b). Thus, the magnitude of wavelength shifts, reflecting chemical or architectural changes in carotenoids, maximized at intermediate K_2TeO_3 concentrations. Further investigation should evaluate the hypothesis that a carotenoid-based detoxification response is invoked by K_2TeO_3 until oxidative stress above 200 µg/ml overwhelms the cell's ability to deal with the toxin via a pigment-based strategy.

After a steep initial decline upon exposure to K_2TeO_3 at 50 µg/ml, BChl content (0.7 nmol/mg dry weight) increased slightly (0.9 nmol/mg dry weight) up to a K_2TeO_3 concentration of 500 µg/ml (Fig. 7.1c). Especially interesting are the inverse trends of BChl and carotenoids between 50 and 200 µg/ml: unlike BChl, carotenoids declined (62 to 26 nmol/mg dry weight) with increasing K_2TeO_3 in the medium. Because the BChl/carotenoid ratio in any given LH or RC complex is fixed, these results imply that pigments in different cellular pools respond in a different way to K_2TeO_3. Cellular BChl content measured by extraction may mask information about the distribution of this pigment among membrane protein complexes, because the solvent removes BChl from the RC, LHI and LHII complexes, and pools the signal regardless of its source.
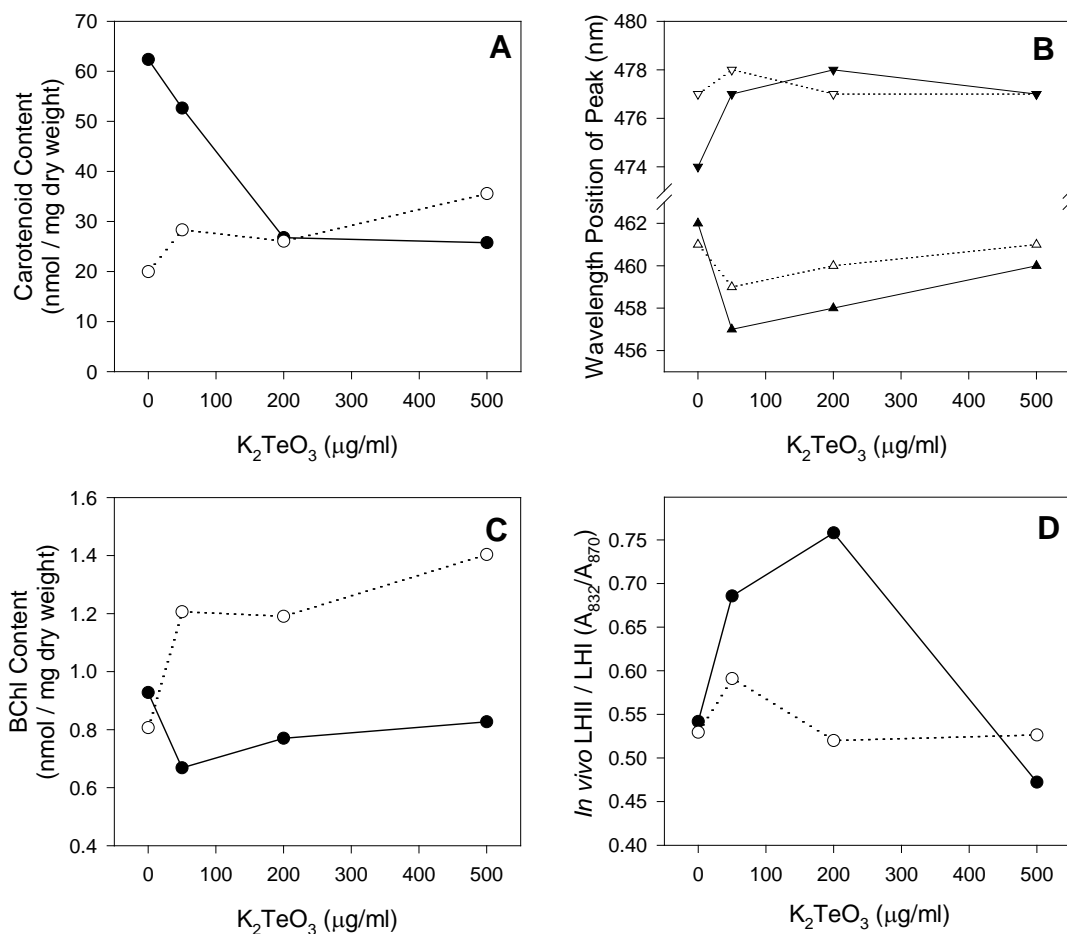
Fig. 7.1. Carotenoid and BChl production in *E. ramosum*, strain E5 cultured in the presence of a range of K_2TeO_3 concentrations and two nutrient levels: rich organic (filled symbols) and oligotrophic (open symbols). **(A)** Dominant carotenoid ($\lambda_{max} = 477$ nm), standardized to biomass. **(B)** Position of the two dominant carotenoid absorption peaks (– ▲ – and –▼–, respectively). **(C)** BChl ($\lambda_{max} = 770$ nm), standardized to biomass. **(D)** Ratio of BChl in LHII ($\lambda_{max} = 798, 832$ nm) to BChl in LHI ($\lambda_{max} = 870$ nm). (A), (B), (C), from acetone/methanol (7:2) extract; (D), *in vivo*.

Investigation of *in vivo* pigmentation in E5 revealed an additional layer of the response of BChl to K_2TeO_3.

Expression of the LHII complex (λ_{max} = 798, 832 nm) relative to the LHI complex (λ_{max} = 870 nm) was maximal at a K_2TeO_3 concentration of 200 μg/ml (Fig. 7.1d). In purple nonsulfur bacteria, LHII complexes are induced under low light conditions (Dubbs and Tabita, 2004), but the nature of their regulation in AAP has not been elucidated. Interestingly, there is evidence that carotenoids in LHII complexes possess especially important roles in oxidative defense in anoxygenic phototrophs, in addition to their light-harvesting activity (Limantara *et al.*, 1998). Efficient quenching of the triplet state of BChl by spheroidene incorporated into LHII of *R. sphaeroides* reduced the yield of BChl cation radicals to half of that generated in the RC complex (Limantara *et al.*, 1998). In E5, LHII may be up-regulated under tellurite-exposure for its carotenoid component rather than its BChl. Although the photosynthetically uncoupled pool of carotenoids in the cell envelope of AAP have been proposed to be utilized in oxidative defense (Yurkov and Beatty, 1998a; Beatty, 2002), LHII carotenoids have not been implicated this way.

7.4.2.2. Nutrient level

Despite a preference for eutrophic habitats, several AAP increase pigment expression when organics are in short supply. In betaproteobacterial *Roseateles depolymerans*, carbon limitation transcriptionally induces the photosynthetic apparatus (Suyama et al., 2002), reflecting an adaptation to exploit oligotrophic conditions in which strict heterotrophs' competitive ability is compromised (Beatty, 2002). Resistance to K_2TeO_3

also increases under nutrient limitation. Yurkov *et al.* (1996) showed that E5 perceived K_2TeO_3 as less toxic in minimal acetate than RO medium. The K_2TeO_3 concentration that induced maximal detoxifying reduction was twenty times higher (1000 μg/ml versus 50 μg/ml) oligotrophically than eutrophically and the MIC correspondingly rose from 750 to 2300 μg/ml. Moore and Kaplan (1992) similarly reported that purple nonsulfur *R. rubrum* and *R. sphaeroides* tolerated higher concentrations of metal(loid)s in the absence of yeast extract. However, until now, research has not addressed the combined effect of nutrients and metal(loid)s on photosynthetic apparatus expression in anoxygenic phototrophs.

In E5, the magnitude of K_2TeO_3-induced effects showed a strong dependence on nutrient concentration. Decreasing the availability of reduced carbon in the medium by a factor of five (from 3 to 0.6 g/l) changed the effect of K_2TeO_3 from inhibitory to stimulatory on the expression of BChl and carotenoids. Under these conditions, E5 resembled the other AAP described in Table 7.1. Analysis revealed four main trends in the response of the photosynthetic apparatus to K_2TeO_3 and nutrients.

First, although 500 μg/ml K_2TeO_3 depressed cellular carotenoids by 58% in organic rich cultures, it induced a nearly two-fold increase under nutrient limitation (Fig. 7.1a). Second, the separation between major carotenoid absorbance peaks underwent a smaller K_2TeO_3-induced wavelength shift oligotrophically (3 nm) than eutrophically (8 nm) (Fig. 7.1b). Third, with K_2TeO_3, BChl content (0.9 nmol/mg dry weight) decreased by 11% in RO cultures (0.8 nmol/mg dry weight), but increased 1.7-fold (from 0.8 to 1.4 nmol/mg dry weight) in low organic cultures over K_2TeO_3-free controls (Fig. 7.1c). Fourth, carbon limitation abolished the 1.5-fold increase in the LHII/LHI ratio that

occurred at intermediate K_2TeO_3 concentrations (50 to 200 µg/ml) in RO culture (Fig. 7.1d).

These results could indicate that E5 employs carotenoids and BChl in a pigment-based defense strategy against low to moderate oxidative stress. Because E5 perceives K_2TeO_3 as less toxic under nutrient limitation (Yurkov et al., 1996), oligotrophic and eutrophic culture conditions serve as proxies for relatively low and high toxicity, respectively. Under low toxicity, both BChl and carotenoids were up-regulated in concert, possibly providing a barrier against comparatively mild oxidative stress. Under higher perceived toxicity, pigment content dropped and the reduction of K_2TeO_3 became the dominant means of detoxification. Interestingly, BChl (especially incorporated into LHII), was more robust to K_2TeO_3-induced oxidative stress than were carotenoids, and it did not suffer the same decline until a K_2TeO_3 concentration of 200 µg/ml.

7.4.3. *E. litoralis*, T4: Elevation of carotenoids, bacteriochlorophyll and its precursors

Strain T4, from a marine supralittoral microbial mat of fluctuating salinity on the island of Texel (Yurkov et al., 1993b; 1994a), demonstrated an even more elaborate response of pigments – especially BChl – to tellurite exposure. T4 also has a well-characterized carotenoid composition (Yurkov et al., 1994a), making it a good candidate for investigation because baseline data on its pigment system exists for comparison.

Thin layer chromatography, followed by spectrophotometry, allowed a more detailed and quantitative analysis of the effects of K_2TeO_3 (500 µg/ml) on synthesis of

various pigments. The method was more sensitive than spectrophotometric analysis of a bulk extract because it permitted distinction of different pigment components, especially those carotenoids and bacteriochlorins that absorbed weakly enough to be obscured in mixtures. Eighteen colored bands were distinguished in a Te-free culture, and 20 bands were obtained from a K_2TeO_3-amended (500 µg/ml) culture (Fig. 7.2a). Disparity in component responses implied that K_2TeO_3 acted via more than one regulatory system.

7.4.3.1. Carotenoids

Components 9 to 14 constituted carotenoids, with absorbance maxima from 453 to 489 nm (Table 7.2). Among these, the major components were identified by comparison of absorbance spectra with previous analysis of the pigment suite of T4 (Yurkov *et al.*, 1994a). Tellurite considerably increased most pigments. The most abundant and least polar pigments (component 14, λ_{max} = 453, 477 nm; component 13, λ_{max} = 473 nm) increased 1.8-fold and 3.1-fold, respectively. Both were attributed to β-β-carotene, with slight differences in structure likely due to, for example, partial retention of lipids. Zeaxanthin (component 9, λ_{max} = 474, 502 nm) and spirilloxanthin (component 12, λ_{max} = 467 nm) were enhanced by factors of 3.4 and 3.0, respectively (Table 7.2). Although the absorbance of erythroxanthin sulfate was obscured by component 1 (λ_{max} = 414 nm), this highly polar carotenoid appeared to increase in the presence of tellurite (Fig. 7.2a). Not all carotenoids were stimulated: bacteriorubixanthinal (component 11, λ_{max} = 489 nm) and pink component 10 (λ_{max} = 477 nm) diminished to 60% and 90% of their Te-free levels, respectively. This disparity in response strengths is congruent with the peculiar

Fig. 7.2. Influence of K_2TeO_3 on pigment synthesis in *E. litoralis*, strain T4. **(A)** Pigments separated by thin layer chromatography; solvent system: petroleum ether/diethyl ether/actone (40:10:10). Lanes were loaded with pigment volumes proportional to biomass. Left lane, K_2TeO_3-free; right lane, amended with 500 μg/ml K_2TeO_3; Central numerals, components common to both treatments; numerals adjacent to one lane, components isolated from only the respective treatment. **(B)** Schematic of partial BChl synthesis pathway, showing where K_2TeO_3 likely exerts inhibitory effects (Ouchane *et al*., 2004). Numerals, characteristic absorption peaks of components.

Table 7.2. Influence of 500 µg/ml K_2TeO_3 on absorption characteristics of pigments isolated from strain T4 by thin layer chromatography.

Pigment Comp.	Peak Wavelength (nm)		Increase Factor	Peak shift (nm)	Color	Identity
	- tellurite	+ tellurite				
1	**414**, 501, 539, 585, 629	**416**, 498, 548, 586	2.2	2	brown	N.D.
1C		416, 504, 549, 587, 626	New	0	magenta	N.D.
1A	**414**, 502, 538, 585, 629, 673, 748	**415**, 502, 549, 587, 658, 748	1.6	1	pale bluish	N.D.
2	**416**, 503, 549, 588, 630, 749	**416**, 550, 588, 748	1.2	0	lavender	N.D.
3	**416**, 551, 589	**416**, 550, 588	2.0	0	magenta	MgP or MgPME
3B	414, 540, 588		Abolished		yellowish-orange	N.D.
4	**411**, 580, 634, 678, 756	**419**, 503, 679, 768	3.1	8	brownish-orange	N.D.
5	592, **770**	367, 396, 591, **770**	2.4	0	blue	Bacteriochlorophyll
6	416, **680**, 759	419, 439, **680**, 754	1.5	0	green	N.D.
6B		439, 679, 764	New		pale orange	N.D.
7	414, **677**	358, 388, 451, **679**, 751	2.2	2	pale orange	N.D.
8	357, 384, 523, 677, **746**	358, 385, 473, 678, **746**	0.9	0	pink	Bacteriopheophytin
9	358, **474**, 502	362, **474**	3.4	0	bright orange	Zeaxanthin
10	**455**, 477	**457**, 480	0.9	2	pink	N.D.
11	**489**	**479**	0.6	-10	deep purple	Bacteriorubixanthinal
12	**467**	**461**	3.0	-6	pinkish purple	Spirilloxanthin
13	**473**	**473**	3.1	0	bright yellow	B-B-carotene
14	**453**, 477	**453**, 476	1.8	0	bright yellow	B-B-carotene

Pigment Comp., pigment component, as in Fig. 7.2a. Negative peak shift, blue-shift; positive peak shift, red-shift. Peak shift reported for peaks in boldface. N.D., Not determined. MgP or MgPME, Mg protoporphyrin or its monomethyl ester. Components listed by decreasing order of polarity.

distribution of carotenoids into two suites in AAP. One pool (erythroxanthin sulfate in *E. longus* and the majority of carotenoids in *E. ramosum* and *Roseococcus thiosulfatophilus*; Noguchi *et al*., 1992; Yurkov *et al*., 1993a) is not bound to the BChl complexes of the photosynthetic RC, but rather to the envelope fraction, consisting of the cytoplasmic membrane and cell wall. The response of these carotenoids that are disengaged from light energy transduction should not necessarily conform stoichiometrically to those that are RC- and LH-integrated. The role(s) of non-photosynthetic carotenoids in AAP have only been speculated upon, but their comparative abundance hints at an important purpose. Indeed, erythroxanthin sulfate is one of the dominant carotenoids in *E. longus* and the most abundant pigment in *E. ramosum* and *E. litoralis* (Noguchi *et al*., 1992; Yurkov *et al*., 1993a; Yurkov *et al*., 1994a). A leading hypothesis is mitigation of photo-oxidative stress (Beatty, 2002). Illumination of BChl generates its triplet state, which in anaerobic phototrophs is quenched by carotenoids before it leads to highly oxidizing reactive singlet oxygen species (Ouchane *et al*., 1997; Glaeser and Klug, 2005; Britton, 2008). It is not surprising that the exclusively aerobic AAP are endowed more richly with antioxidant carotenoids, but their *in vivo* protective functions have not yet been studied in AAP, including possible amelioration of K_2TeO_3-induced oxidative stress. This effect would help explain the preponderance of constitutive high level metal(loid) resistance in AAP (Yurkov *et al*., 1996). If respite from oxidative stress is indeed a prominent role of photosynthetically detached carotenoids, then it is reasonable to expect that K_2TeO_3 could induce their over-expression.

Some carotenoids in T4 also exhibited wavelength shifts in the presence of K_2TeO_3, as revealed by TLC, which permitted the investigation of separate pigments.

Both bacteriorubixanthinal (component 11, λ_{max} = 489 nm) and spirilloxanthin (component 12, λ_{max} = 467 nm) were blue-shifted in the presence of 500 µg/ml K_2TeO_3, by 10 and 6 nm, respectively. Several carotenoids, including zeaxanthin (abundant in T4), can form aggregates in natural systems, leading to blue or red peak shifts depending on the molecular configuration of the aggregates (Köhn *et al.*, 2008). Carotenoid aggregates within the lipid bilayer are known to protect membrane lipids from peroxidation caused by oxidizing agents (Köhn *et al.*, 2008). Carotenoids in the cell envelope of AAP are well positioned to fill this role. For example, zeaxanthin molecules are long enough to span the membrane and strengthen it rivet-like (Britton, 2008). Zeaxanthin also possesses polar end groups, which are inserted into the hydrophilic portion of the membrane and protect against oxidative damage from radicals in the aqueous phase (Britton, 2008). Hence, whereas zeaxanthin did not exhibit spectrophotometric signs of aggregation in T4, (λ_{max} = 474 nm with and without K_2TeO_3), this carotenoid may nevertheless provide structural protection against membrane oxidation. Although carotenoid radicals generated by oxidizing agents also exhibit absorbance shifts (Köhn *et al.*, 2008), this process is unlikely to explain λ_{max} changes in carotenoids of T4. Carotenoid radicals frequently display absorbance shifts of several tens of nm (El-Agamey and McGarvey, 2008), rather than the 1-10 nm peaks shifts in T4 carotenoids.

7.4.3.2. Bacteriochlorophyll and bacteriopheophytin

Absorbance at 770 nm (component 5) confirmed the presence of BChl *a* (Table 7.2). Tellurite increased BChl 2.4-fold. Tellurite may stimulate BChl for at least three reasons.

First, BChl may be fortuitously overexpressed if K_2TeO_3 acts on the promoter for the entire photosynthetic superoperon, even if only carotenoids are involved in defense. Second, BChl may work in concert with carotenoids to scavenge radicals generated by exposure to K_2TeO_3, because transient carotenoid radicals react very efficiently with porphyrins such as Chl or pheophytin via electron transfer (El-Agamey and McGarvey, 2008). This strategy would deepen the cell's electron sink and free carotenoids for more efficient radical scavenging. Third, the photosynthetic apparatus may be up-regulated in response to metal(loid)-detoxification associated energetic stress placed upon the cell. Such a response may reflect the tendancy of AAP to increase photosynthetic pigment expression under energetically stressful conditions such as starvation or suboptimal pH/salinity/temperature, presumably to supplement their depleted store of cellular energy with input from the illumination (Rathgeber *et al.*, 2004; Yurkov and Csotonyi, 2009). This would enhance their competitive potential in the community.

Abundance of the pinkish pigment bacteriopheophytin (BPhe) (component 8, λ_{max} = 746 nm), an integral component of the RC, was not appreciably affected by K_2TeO_3 (factor of 0.93) (Table 7.2). Because of the fixed stoichiometric ratio of BChl and BPhe in the RC, this observation implies that the RC complex is relatively unaffected by K_2TeO_3, and that only peripheral LH complex pigments or the photosynthetically disengaged carotenoid pool are susceptible to K_2TeO_3.
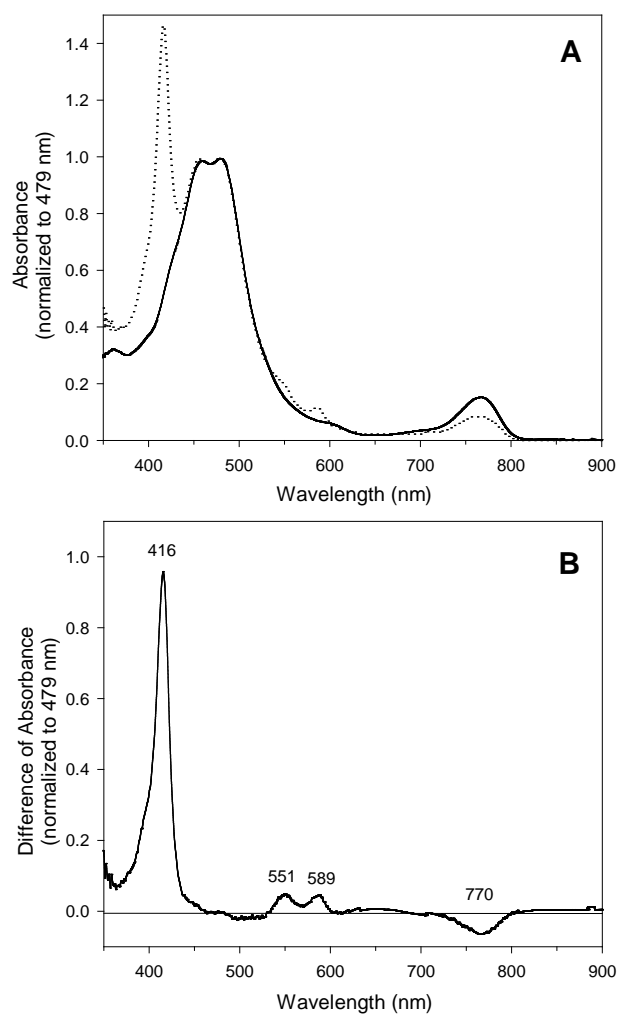
Fig. 7.3. Induction of BChl precursor by K_2TeO_3 in *Erythrobacter litoralis*, strain T4. **(A)** Absorption spectra from acetone/methanol (7:2) extract in the presence of K_2TeO_3 (1000 µg/ml) (⋯⋯) and in its absence (——). **(B)** Difference spectrum of T4 from absorption spectrum traces in (A), demonstrating novel peaks and concomitant deficiency of BChl induced by K_2TeO_3. Numerals above absorbance spectra, λ_{max} values.

7.4.3.3. Bacteriochlorophyll precursors

Several relatively polar components (compounds 1, 1C, 1A, 2, 3, 3B, 4) had absorbance spectra resembling precursors of BChl (Table 7.2). All possessed a major peak between 411 and 419 nm, corresponding to the porphyrin ring in BChl precursors such as MgP (Kamogawa *et al*., 1989). The most strongly absorbing compound in the pigment profile of T4 possessed a λ_{max} of 416 nm, with minor peaks at 551 and 589 nm (Fig. 7.3a) (Table 7.2), consistent with MgP or its monomethyl ester (with absorption maxima at 416, 552 and 590 nm; Ouchane *et al*. 2004). Whereas BChl precursors, including MgP, have been isolated from anoxygenic phototrophs such as *Rhodobacter sphaeroides* (Jones, 1963a) and *R. gelatinosus* (Ouchane *et al*., 2004), none have been reported from AAP. When T4 was cultured at 1000 µg/ml K_2TeO_3, near the MIC of the strain (1200 µg/ml; Yurkov *et al*., 1996), a 416-nm component was more strongly expressed than in metalloid-free cultures (Fig. 7.3a). A difference spectrum between acetone/methanol (7:2) extracts of K_2TeO_3-amended and Te-free treatments revealed that (1) absorbance peaks of 416, 551 and 589 nm matched both those of component 3 from TLC analysis (Fig. 7.5) and the absorbance spectrum of a standard MgP solution (Frontier Scientific), and (2) its presence was associated with depleted BChl (Fig. 7.3b).

A two-fold stimulatory effect of 500 µg/ml K_2TeO_3 on component 3 was recorded using TLC (Table 7.2). The observed elevation of both BChl and MgP in the presence of K_2TeO_3 at first appeared to contradict their previously recorded inverse relationship (Fig. 7.3a). However, culture kinetics indicated that MgP initially accumulated to a maximum level (Fig. 7.4b) only during exponential growth of K_2TeO_3-amended cultures (Fig. 7.4a).
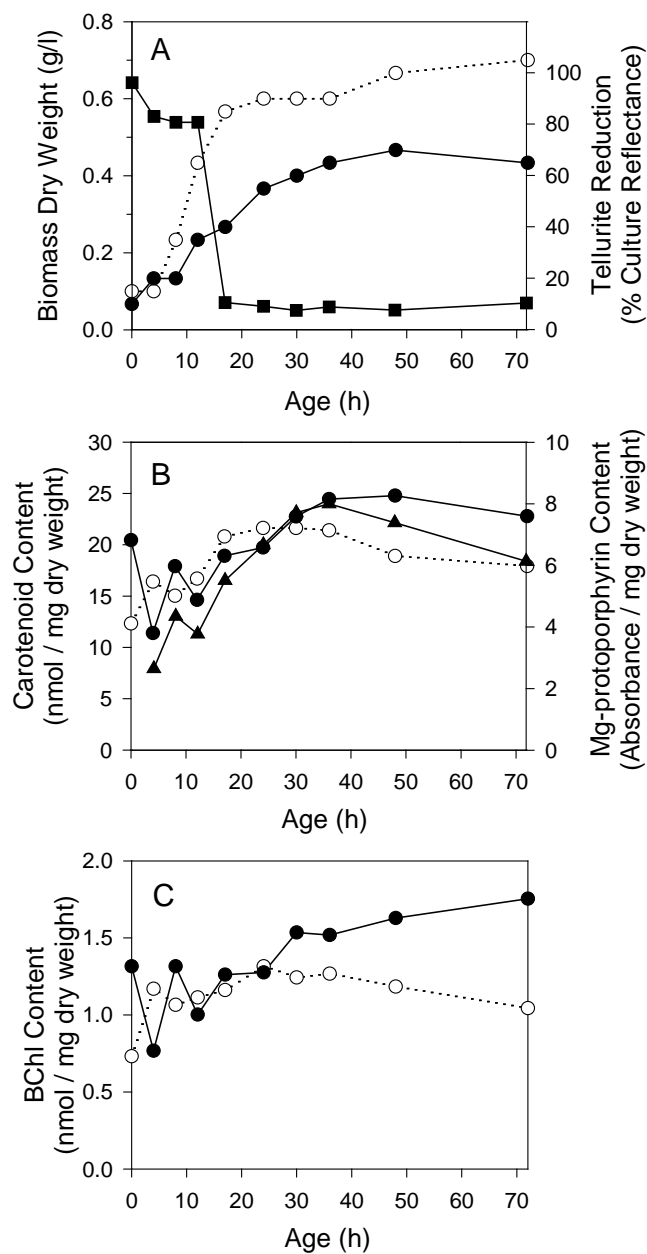
Fig. 7.4. Kinetics of influence of K_2TeO_3 on pigment synthesis in *E. litoralis* exposed to 0 (open symbols) and 200 µg/ml (filled symbols) of K_2TeO_3. **(A)** Biomass (–●–), and K_2TeO_3-reduction (–■–) determined from culture reflectance. **(B)** Major pigments from acetone/methanol extract: carotenoids (λ_{max} = 479 nm) (–●–) and MgP or MgPMe (λ_{max} = 416 nm) (–▲–). **(C)** BChl from acetone/methanol extract (λ_{max} = 770 nm).

However, MgP declined in stationary phase much of it probably converted to BChl (Fig. 7.4c), so that the metalloid-amended treatment finally expressed 1.3 times as much BChl as the metalloid-free culture. Relative concentrations of BChl and MgP therefore depended on culture age.

Six additional components with absorbance characteristics implicating them as BChl biosynthesis intermediates accumulated in either Te-free (components 1, 1A, 2, 3B, 4) or K_2TeO_3-amended (components 1, 1C, 1A, 2, 4) cultures. Distinguished on the basis of their chromatographic separation (Fig. 7.2a) and absorption spectra (Fig. 7.5; Table 7.2), the components appeared different from precursors of BChl reported from *R. sphaeroides* (Jones, 1963a; 1963b, Lascelles et al., 1966) and *R. gelatinosus* (Ouchane *et al.*, 1997; 2004). Components 1, 1C, 1A and 4 shared absorption peaks with protoporphyrin IX (the immediate precursor to MgP, with maximal absorbance at 402, 504, 538, 574 and 680 nm; Ouchane *et al.*, 2004), but some peaks were missing (e.g. 402 nm), and new ones were present (e.g. 748 or 749 nm in components 1A and 2), indicating some structural distinction (Fig. 7.5; Table 7.2). Tellurite also abolished the yellowish-orange component 3B ($\lambda_{max} = 414, 540, 588$ nm), but induced a novel magenta colored component, 1C ($\lambda_{max} = 416, 504, 549, 587, 626$ nm), whose absorbance spectrum somewhat resembled that of MgP (Fig. 7.5; Table 7.2). Finally K_2TeO_3 stimulated (by factors of 1.5 and 2.2) and altered the absorbance spectrum of two pigments (green component 6, $\lambda_{max} = 416, 680, 759$ nm and pale orange component 7, $\lambda_{max} = 414, 677$ nm, respectively) that were less polar than BChl. Tellurite also induced the novel expression of a third pale orange component (6B, $\lambda_{max} = 439, 679, 764$ nm) (Table 7.2),
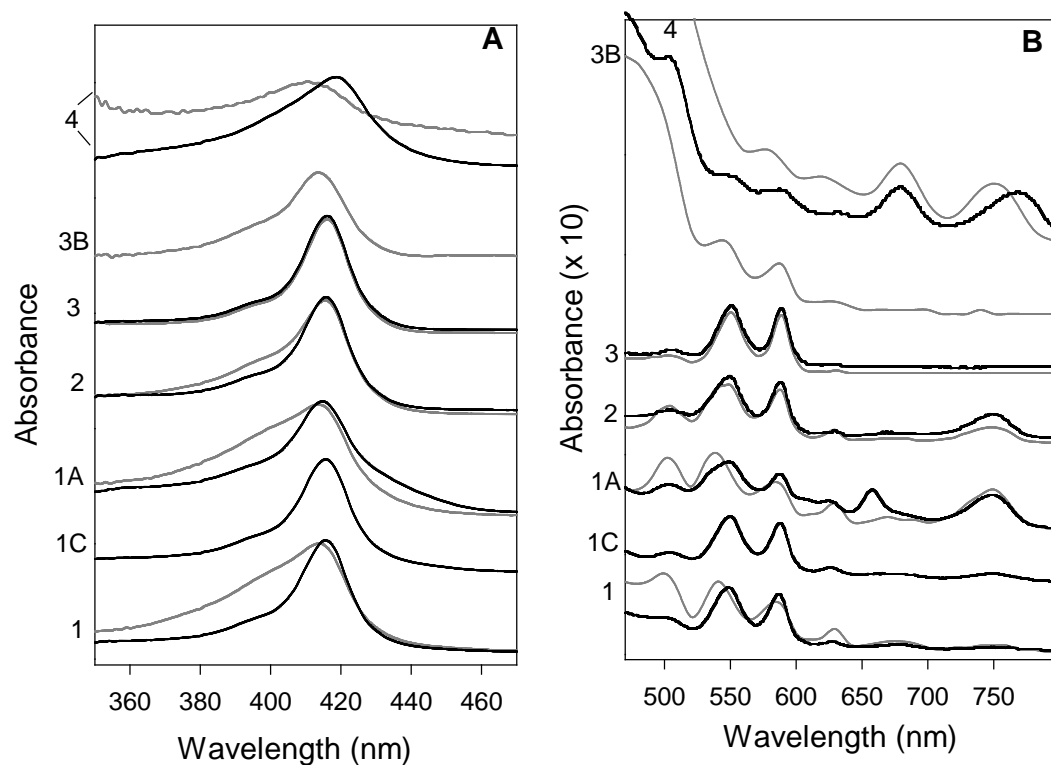
Fig. 7.5. Putative BChl intermediates generated in the absence (grey lines) and presence (black lines) of 500 μg/ml K_2TeO_3, standardized to absorbance of Soret peak. **(A)** Blue region of visible light spectrum. **(B)** Red section of visible light spectrum. Numerals, individual pigment components from Fig. 7.2a.

In K_2TeO_3-amended cultures, all components were elevated above those in metalloid-free cultures, with factors ranging from 1.2 (component 2) to 3.1 (component 4) (Table 7.2). Exposure to tellurite also caused shifts in peak wavelengths and altered relative absorbance. Such effects were most obvious in (1) the 4 nm blue-shift and elevated absorbance of the 537 nm peak of component 2 along with a greatly elevated absorbance at 748 nm; (2) the 11 nm red-shift at 538 nm, and elevation of the 502-nm peak of component 1A; (3) disappearance of a 501-nm peak and a 9-nm red-shift of the 539-nm peak of component 1; and (4) a 12-nm red-shift of component 4 at 756 nm (Fig. 7.5; Table 7.2). In fact, the only pigment whose absorbance spectrum remained congruent with or without K_2TeO_3 was component 3(MgP or MgPMe) (Fig. 7.5). This implies that MgP is the most structurally robust BChl intermediate in the presence of K_2TeO_3.

Investigation of several halophilic AAP from a brine spring (Csotonyi *et al*., 2008) revealed metal(loid)-induced expression of additional putative BChl precursors (Fig. 7.6). Strains EG7, EG8 and EG13 were all members of the α-3 proteobacterial *Roseobacter* clade, unlike the α-4 *Proteobacteria* in most of this work. Their response demonstrated that interruption of BChl synthesis by metal(loid)s is widely distributed throughout AAP, and is caused by Na_2SeO_3 as well as K_2TeO_3 (Fig. 7.6c, d). Strains EG7, EG8 and EG29 expressed substantially less BChl and carotenoids when grown with only 50 μg/ml K_2TeO_3 or up to 1000 μg/ml Na_2SeO_3. In EG29, K_2TeO_3 was twenty times more toxic to pigment expression than was Na_2SeO_3 (Fig. 7.6e, f). Metavanadate had little impact on the suite of pigments expressed, but in strains EG7 (Fig. 7.6a, b) and
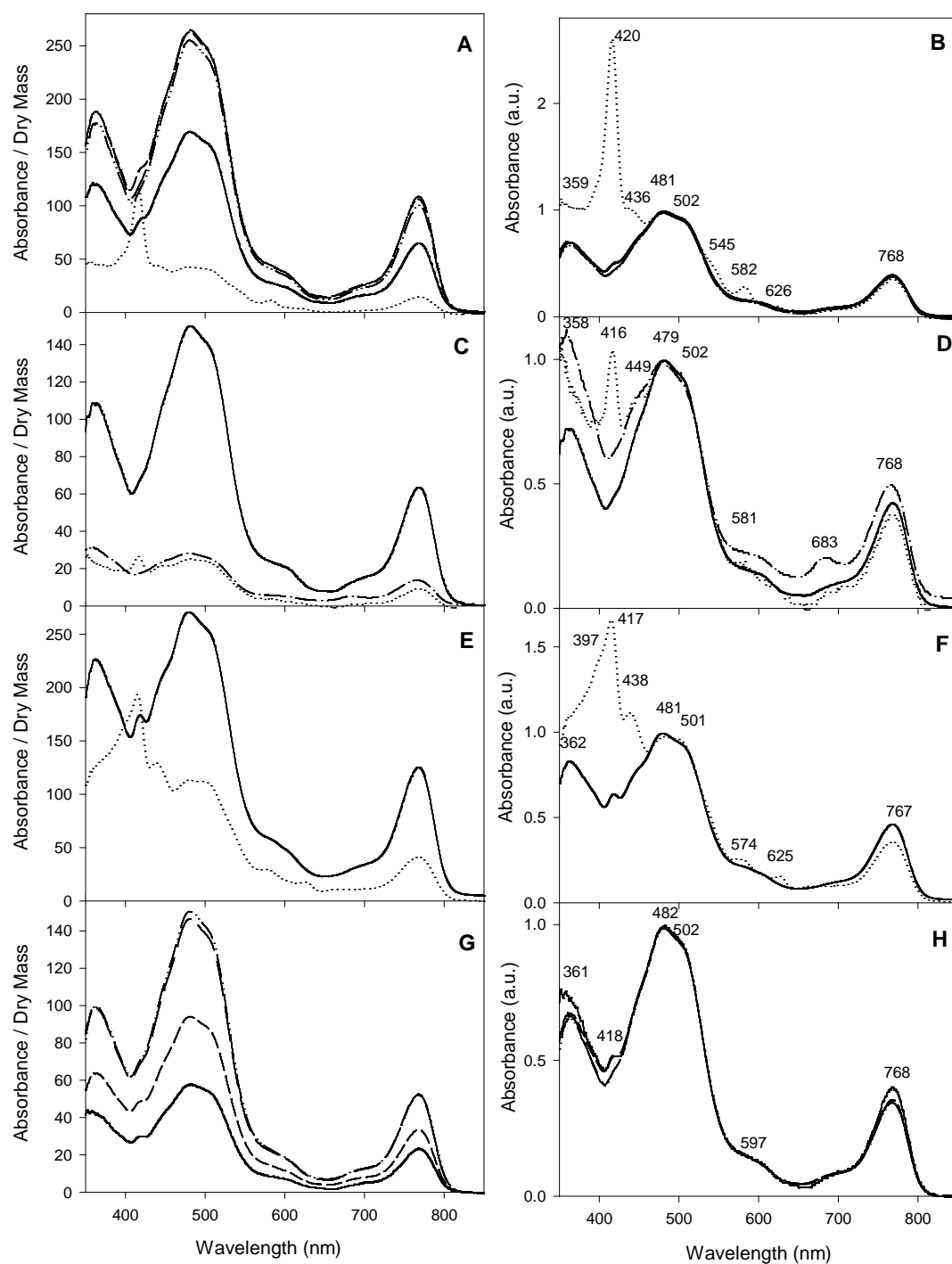
Fig. 7.6. Absorption spectra from acetone/methanol extracts of four brine spring AAP cultured without metal(loid)s (—) or with various concentrations (μg/ml) of K_2TeO_3, Na_2SeO_3 and $NaVO_3$. Left column, absorbance normalized to cellular biomass; right

column,absorbance normalized to dominant carotenoid (λ_{max} = 480 nm); legends apply to both left and right panels. **(A, B)** Pinkish-purple EG7 with K_2TeO_3 (⋯⋯, 50) and $NaVO_3$ (- - -, 50; —— —, 500, –··– 1000). **(C, D)** Pinkish-orange EG8, with K_2TeO_3 (⋯⋯, 50) and Na_2SeO_3 (–··–, 1000). **(E, F)** Pinkish-purple EG29, with K_2TeO_3 (⋯⋯, 50). **(G, H)** Pinkish-orange EG13, with $NaVO_3$ (- - -, 50; —— —, 500, –··– 1000). Numerals above absorbance spectra, λ_{max} values.

EG13 (Fig. 7.6g, h), 50 to 1000 μg/ml $NaVO_3$ markedly stimulated pigment expression, similar to the effect of K_2TeO_3 on T4 and JF1 (Tables 1, 2).

BChl precursors might accumulate in AAP for several reasons. In the absence of K_2TeO_3 they are apparently induced by low aeration, probably because of partial inhibition of one or more O_2-requiring enzymes, analogous to AcsF in the purple nonsulfur bacterium *R. gelatinosus*, where it catalyzes the conversion of MgPMe to Mg-divinyl protochlorophyllide *a* (Ouchane *et al*., 2004). Its inactivation results in accumulation of MgPMe at the expense of BChl (Ouchane *et al*., 2004).

The question of why tellurite induces additional accumulation of BChl precursors such as MgP or MgPMe is best separated into proximate and ultimate components: (1) by what mechanism BChl precursors accumulate, and (2) what purpose their accumulation serves. There are two plausible answers to the first question. First, K_2TeO_3-induced oxidative stress inhibits enzymes catalyzing BChl biosynthesis (Fig. 7.2b), much like methyl viologen induces hyperaccumulation of MgPMe and protoporphyrin IX in plants (Chereskin *et al*., 1982; Aarti *et al*., 2006). Indeed, the increased accumulation of component 3 (MgP or MgPMe) implies that K_2TeO_3 may interrupt the BChl biosynthesis pathway by inhibiting enzymes catalyzing the chelation of protoporphyrin IX with Mg to form MgP, or the methylation of MgP to MgPMe (Fig. 7.2b). Second, K_2TeO_3 may elicit symptoms of Fe deficiency, as does Mn in the cyanobacterium *A. nidulans* (Csatorday *et al*., 1984). Due to its similarity in size to Fe, tellurite could compete with Fe for binding sites on enzymes such as AcsF, the gene for which encodes a binuclear-iron-cluster-containing protein (Pinta *et al*., 2002). In purple nonsulfur bacteria, cyanobacteria and plants, Fe is also required for the formation of MgP (Lascelles, 1966). Because oxidative

stress and Fe-replacement hypotheses are not mutually exclusive, K_2TeO_3 may mediate tetrapyrole accumulation in AAP by both processes. Although photooxidative stress in *R. gelatinosus* leads to mutants that accumulate BChl precursors (Ouchane *et* al. 1997), mutation in photosynthesis genes does not explain overexpression of MgP in T4, because BChl production is not abolished completely as it was in *R. gelatinosus*.

Whether BChl precursors have a functional role in K_2TeO_3-exposed T4 is more difficult to answer. The problem lies in their photodynamic nature (Larkin and Chory, 2006). Free porphyrin intermediates such as protoporphyrin IX are more likely to induce oxidative stress than BChl incorporated into pigment-protein complexes, which is in close association with triplet-state quenching carotenoids (Glaeser and Klug, 2005). Protoporphyrin IX enhances cells' susceptibility to oxidative stress partly by facilitating light-induced lipid peroxidation (Williams *et al*., 1994). Mutants of *E. coli* in the *visA* gene, which encodes ferrochelatase (an enzyme that consumes protoporphyrin IX in the heme biosynthesis pathway), die when exposed to light. Cells accumulate protoporphyrin IX, which produces a reactive oxygen species upon illumination (Nakahigashi *et al*., 1991). Some metal chelated porphyrins, such as Sn- and Zn- protoporphyrin, are also potent prooxidants (Fang *et al*., 2003; Uc *et al*., 2007). Surprisingly however, porphyrin intermediates may become antioxidant under certain conditions. Protoporphyrin IX actually inhibits lipid peroxidation in the dark by scavenging peroxyl radicals in a reaction that degrades the pigment (Williams *et al*., 1994). This dark antioxidant role is relatively more significant for AAP than for anaerobic anoxygenic phototrophs because AAP synthesize BChl in the dark, potentially providing them a unique opportunity to utilize porphyrin intermediates in oxidative defense.

Unfortunately, not enough is known about the pro/antioxidant tendancy of MgP or MgPMe. Therefore, it is impossible to state conclusively whether these tetrapyrole intermediates are deleterious or beneficial to T4. Porphyrins accumulating via the enzyme-inhibiting effects of K_2TeO_3 may scavenge radicals generated by K_2TeO_3, thus protecting the plasma membrane in a kind of autoregulatory way against the very metalloid that induced them. Because of the propensity for transfer of radical electrons between carotenoids and porphyrins (El-Agamey and McGarvey, 2008), these components may act in concert to quench oxidatively reactive chemical species. Alternatively, if some porphyrin intermediates are prooxidant even in the dark, then carotenoid over-expression may be the cell's response to porphyrin-induced reactive oxygen species. Even if porphyrins are prooxidant, they may fill crucial communicative roles. MgP functions as a retrograde (chloroplast to nucleus) signal molecule under stressful conditions to down-regulate photosynthetic apparatus genes encoded on the nuclear chromosome (Kanesaki *et al.*, 2009). Future studies may test whether MgP signals the cell to mount defense against tellurite via reductases or carotenoids.

In summary, AAP are highly derived descendents of anaerobic anoxygenic phototrophic bacteria, not only in their strictly photosynthetic characteristics, but also in their nearly unexplored intrinsic high level resistance to metal(loid)s (Yurkov *et al.*, 1996). In this work, we have begun to unravel the complexities of the interaction of AAP photosynthetic pigment systems with the chalcogen oxyanion tellurite. Oddly, in several species, this toxin stimulated increased pigment production. The strategy implies an inducible defense mechanism, because over-expression of energetically costly pigments

in organisms that are already metabolically stressed by a potent oxidizing agent would be wasteful and therefore competitively disadvantageous. In this way, AAP differ markedly from purple nonsulfur bacteria such as *R. sphaeroides*, which possesses carotenoids with a demonstrated antioxidant role, but which fails to up-regulate its carotenoids upon exposure to photooxidative stress (Glaeser and Klug, 2005). Future research must elucidate the mechanisms by which carotenoids, BChl and the precursors of BChl interact in cellular defense against heavy metals in AAP.

7.5. Acknowledgements

7.6. References

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Chapter 8.

Conclusions and Future Perspectives.

8.1. Major findings of the thesis

This study has focused on two principal groups of microorganisms that are pivotal components of extreme environments: (1) anoxygenic phototrophs, which replace eukaryotic oxygenic phototrophs as leading primary producers and modulators of reduced organic carbon under stressful conditions, and (2) metal transforming bacteria, which survive by performing redox reactions to detoxify metallic and semi-metallic oxyanions in their environment. Both groups occupy ecologically influential positions by modifying the availability of resources to other species or by altering the chemical hospitability of the environment. By virtue of their unique characteristics, hypersaline springs and hydrothermal vents have been especially valuable in the quest to understand the phylogenetic and physiological diversity of anoxygenic phototrophs and metal(loid) resistant bacteria.

The first objective of this study was therefore to enumerate the anoxygenic phototrophic community of an unusual hypersaline spring system, East German Creek, that is new to microbiological investigation. The high flow rate and turbulence of the EGC hypersaline springs provided the oxygenated conditions that generated the development of an especially abundant and diverse community of AAP (Table 2.3; Fig. 2.2), including the most halotolerant strains yet reported (Csotonyi *et al.*, 2008). Although obligately anaerobic purple phototrophs related to *Halochromatium* and *Roseospira* were present, AAP made up 16-36% of cultivable bacteria, being some of the highest yet reported and lending further evidence to the extensive adaptation of AAP to extreme environments.

The finding of a plethora of novel AAP at EGC facilitated fulfilment of the second goal of the project, the description of novel species of anoxygenic phototrophs with special

phylogenetic and physiological relevance. A substantial contribution was made to the taxonomy of AAP, for the current project included the description of three new species in two new genera, one of which was an unusual gammaproteobacterial representative. *Chromocurvus halotolerans*, EG19[T] has been proposed as a second known AAP member of the *Gammaproteobacteria* (Fig. 3.3). Its bent cells (Fig. 3.1), reminiscent of spirilla, were unusual among AAP. The investigation of EGC anoxygenic phototrophs also significantly advanced the field of photosynthetic physiology. From the steep redox gradient of the EGC effluent streams was isolated and taxonomic described the first microorganism, *Charonomicrobium ambiphototrophicum*, EG17[T], capable of both aerobic and anaerobic anoxygenic photosynthesis (Fig. 4.3). Straddling the border between AAP and purple nonsulfur bacteria, this novel genus is likely to reveal much about the evolution of the increasingly investigated AAP. An additional surprise of the EGC springs was inexplicably high bacterial resistance to oxyanions of the metal(loid)s Te, Se and V despite the absence of detectable quantities of these elements in the spring water. In fact, one proposed novel AAP species, *Roseovarius vanadiphilum*, EG13[T], exhibited a unique stimulatory response to extremely high concentrations of metavanadate (at least 10 g/l or 82 mM) when cultured on glutamate (Fig. 6.3). Never has a stimulatory effect of V compounds been demonstrated for anoxygenic phototrophs, and in no organism has a beneficial aerobic effect of V been documented at a concentration greater than 0.1 mM (i.e. Meisch and Becker, 1981).

High metal(loid) resistance was much more expected in bacteria isolated from the second type of environment explored by this study, the deep ocean hydrothermal vent ecosystems of the Juan de Fuca and Explorer Ridges, because vent fluids are typically highly enriched in metal(loid)s (Kelley *et al.*, 2002). Although the study did not recover novel anoxygenic phototrophic taxa from this habitat, the third objective of the project was met, for the vent

community yielded the first example of bacterial anaerobic respiration on oxidized Te, as well as the first reports of selenite and metavanadate respiration in this ecotope (Table 5.1; Fig. 5.2) (Csotonyi *et al*., 2006). Experiments on growth kinetics (Fig. 5.3), cellular ATP content (Fig. 5.4) and response to protonophores confirmed that a close phylogenetic relative of *Shewanella frigidimarina* was capable of using tellurate as a sole terminal electron acceptor during anaerobic growth. Close association of metal(loid) respiring strains with vent animals such as sulfide worms (*Paralvinella sulfincola*) (Fig. 5.1) further underscored the importance of the structure of macrobiological communities in determining the distribution of hydrothermal vent microorganisms, and pointed the way to future studies on potential animal/microorganism mutualisms in these deep ocean sites.

The two major physiological facets of the project – phototrophy and metal(loid) reduction – were unified in the final goal of the study: probing the interaction between the two metabolic processes. This endeavour was based on the observation that one group of anoxygenic phototrophs thriving in extreme environments, the AAP, are also among the most highly metal(loid) resistant organisms known to science. Although AAP clearly possess efficient metal(loid) reduction systems (Yurkov, *et al*., 1996), their rich suite of carotenoids have been implicated in photo-oxidative protection (Beatty, 2002). The current investigation represented the first measurement of the influence of tellurite on photosynthetic pigment synthesis, and it focused on a diversity of highly tellurite-resistant AAP (Table 7.1, Fig. 7.6) from both hydrothermal (terrestrial and deep ocean) and hypersaline (brine spring and tidal flat) habitats. The results illustrated that in most species toxic tellurite could surprisingly induce the overexpression of BChl and carotenoids (Table 7.1; 7.2; Fig. 7.1). These findings implicated the abundant photosynthetically disengaged and highly conjugated carotenoids of AAP as

components of a pigment-based oxidative defense mechanism against tellurite toxicity. Interestingly, the study was also the first to confirm the presence of biosynthetic precursors of BChl (e.g. Mg protoporphyrin or its monomethyl ester) in AAP (Fig. 7.3; 7.5). Their detection shed light on the mechanisms by which tellurite likely influences photosynthesis in this group of bacteria.

8.2. Future prospectives

The results of this investigation, while yielding insights into anoxygenic and metal(loid) resistant microbial communities in two unusual extreme environments, by no means satisfy curiosity, but raise a plethora of questions for future research to tackle. Much remains to be explored in the unique hypersaline environment of EGC, starting with a modified enumeration. Although culture-based techniques are useful for subsequent study of the isolated organisms, further investigation should also plumb the microbial diversity of EGC from a molecular biological approach. Quantitative PCR of mat samples using primers developed for specific anoxygenic phototrophic groups would accurately reveal the relative abundance of each functional group. Associating this technique with high-throughput cultivation under selective culture conditions would also facilitate the discovery of additional novel taxa bearing unusual physiological characteristics. A molecular biological approach would also allow determination of the degree of genetic isolation of bacteria from physically separated springs and effluent streams distributed across the EGC playa and between different spring systems in the Lake Winnipegosis region. Such data could take advantage of the geologically young origin of the springs following glacial retreat (Patterson *et al.*, 1997) to estimate the rate of evolution of microbial groups.

Ease of access to the EGC site also makes it desirable for microbial ecological studies. For example, repeated sampling could address the influence of seasonality on anoxygenic phototrophic populations in this strongly continental northern climate. The answer to this question will have great implications to the carbon budget because primary production at EGC is almost exclusively microbial. In particular, because AAP constituted such a large proportion of the cultivable bacterial community, their modulation of organic carbon availability to the balance of the heterotrophic microbial community will likely be substantial. For similar reasons, it will be important to measure the abundance and distribution of organisms simultaneously capable of aerobic and anaerobic photosynthesis (e.g. *Charonomicrobium ambiphototrophicum*). In addition, a full genome sequence of this organism combined with a biophysical investigation of its photosynthetic apparatus would shed light on both its evolution and what features permit the coexistence of two energy transduction pathways that are normally incompatible on the basis of redox potential.

Repeated geochemical sampling of EGC would also address the question of whether the peculiarly high metal(loid) resistance of EGC bacteria is truly constitutive despite the absence of measurable Te, Se and V, or whether the metal(loid) content of spring water rises in transient pulses that maintain selection for resistance in the microbial community. Of particular interest is a better understanding of the beneficial role that metavanadate plays in the physiology of *Roseovarius vanadiphilum*, EG13^T. It is currently unknown whether the oxyanion serves as an electron shuttle, an enzymatic cofactor, or something else entirely.

Although the microbiology of deep ocean hydrothermal vents is already under intensive investigation, many aspects of bacterial ecophysiology remain in the dark. Whereas this project

has not uncovered novel taxa of deep ocean phototrophs, recent research (Beatty *et al*., 2005) illustrates that unexpected groups may yet turn up. For example, purple or green nonsulfur bacteria have not yet been isolated from such habitats, although their flexible photoheterotrophic metabolism should facilitate their survival. The current study has produced the first examples of anaerobic metal(loid) respiration in this ecotope, but very little is known about how commonplace it is, how many other elements are involved, its phylogenetic breadth and its biogeographical distribution on small and large scales. Discovery of tellurate respiring bacteria provides the opportunity to study the enzymology of this novel metabolic mode, as tellurate and tellurite reductases (especially respiratory types) are still uncharacterized. That research would have clear extensions into bioremediation of metal(loid)-contaminated industrial sites. Beyond metal(loid) reduction is the search for hypothetical enzymatic oxidation of Te and V, neither of which is yet documented in any organism. Such a discovery would close the biogochemical cycle for both Te and V. Highly reduced effluents of hydrothermal vents and their entrained metalliferous particles are ideal targets for sampling.

Finally, the interaction between phototrophy and metal(loid) resistance offers myriad directions for additional research to explore. Chief among these is the specific role in oxidative defense played by each pigment whose synthesis is stimulated by tellurite. Whether BChl precursors are incidental or detrimental by-products of oxidative stress, or are actively involved in protective strategies needs to be determined. Why some strains, such as *Erythrobacter litoralis*, T4 exhibit a host of BChl intermediates while closely related organisms such as *Erythromicrobium ramosum*, E5 do not express them is also unknown. This difference may indicate either the presence of a better shielded pigment synthesis pathway in E5 or an interesting porphyrin-based antioxidant mechanism in T4, depending on whether unbound

porphyrins are deleterious or beneficial to the AAP cells, respectively. Furthermore, whereas the results of enhanced synthesis of carotenoids in the presence of tellurite support their antioxidant roles in AAP, the magnitude of this effect has never been quantified. Generation of carotenoid-less mutants such as those for the purple nonsulfur bacterium *Roseivivax gelatinosus* (Ouchane *et al*., 1997) and evaluation of their response to tellurite would facilitate this investigation.

Extreme environments are invaluable to microbiological investigation on both theoretical and applied grounds. Among anoxygenic phototrophs and metal(loid) transforming organisms, the until recently underappreciated AAP offer an especially promising opportunity for future research into both the evolution of major energetic pathways and the development of biotechnological tools.

8.3. References

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