

Aerobic Anoxygenic Phototrophs: Four Decades of Mystery

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Abstract The aerobic anoxygenic phototrophs (AAP) are an important group of bacteria making up large proportions of bacterial communities in both marine and freshwater systems. They thrive in the extreme conditions of hot springs, hypersaline spring systems, and hydrothermal vents and in the presence of high concentrations of toxic metal(loid) oxides. They likely evolved from the purple non-sulfur bacteria, to fill an oxygenated environmental niche, carrying out oxygen-dependant anoxygenic photosynthesis. Investigations into the ecological significance of AAP are in their infancy, although some speculations have now been proposed. Additionally, modern studies are beginning to touch the paradox that is bacteriochlorophyll *a* synthesis in the presence of oxygen as well as the role of abundant carotenoids in AAP. The presence of numerous AAP in every environment tested, in addition to their unique photosynthetic arrangement, are mysteries that have garnered much attention among scientists since their discovery.

Introduction

The aerobic anoxygenic phototrophs (AAP) are a diverse group of bacteria, which produce bacteriochlorophyll (BChl) *a* under oxygenated conditions. The first discovered species, *Roseobacter denitrificans* and *Erythrobacter longus*, were isolated from marine environments almost 40 years ago (Shiba et al. 1979; Shiba and Simidu 1982; Shiba 1991). Soon after that *Sandaracinobacter sibiricus*, *Erythromonas ursincola*, *Erythromicrobium hydrolyticum*, *Erythromicrobium ramosum*, *Erythromicrobium ezovicum*, and *Roseococcus thiosulfatophilus* were found in freshwater hot temperature springs (Yurkov and Gorlenko 1990, 1992a, b; Yurkov et al. 1993a, 1994, 1997). Since then research into AAP has exploded. They have been detected in high abundance in every habitat tested, including oceans, seas, freshwater lakes (Fig. 1a), and river systems around the world, as well as in extreme settings like the phyllosphere (Stiefel et al. 2013), hypersaline spring systems

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(Fig. 1b), hot springs (Fig. 1c), meromictic lakes, hydrothermal vents (Fig. 1d), mine tailings (Fig. 1e), and soil crusts (Fig. 1f) (Rathgeber et al. 2004; Yurkov and Csotonyi 2009; Yurkov and Hughes 2013).

Unlike other anoxygenic phototrophs, AAP require oxygen for both growth and photosynthetic electron transport. They are obligate heterotrophs able to supplement their energy generation with photosynthesis, which physiologically makes them photoheterotrophs and gives them a competitive advantage against other heterotrophs. The main light-harvesting pigment is BChl *a*, which is incorporated into reaction

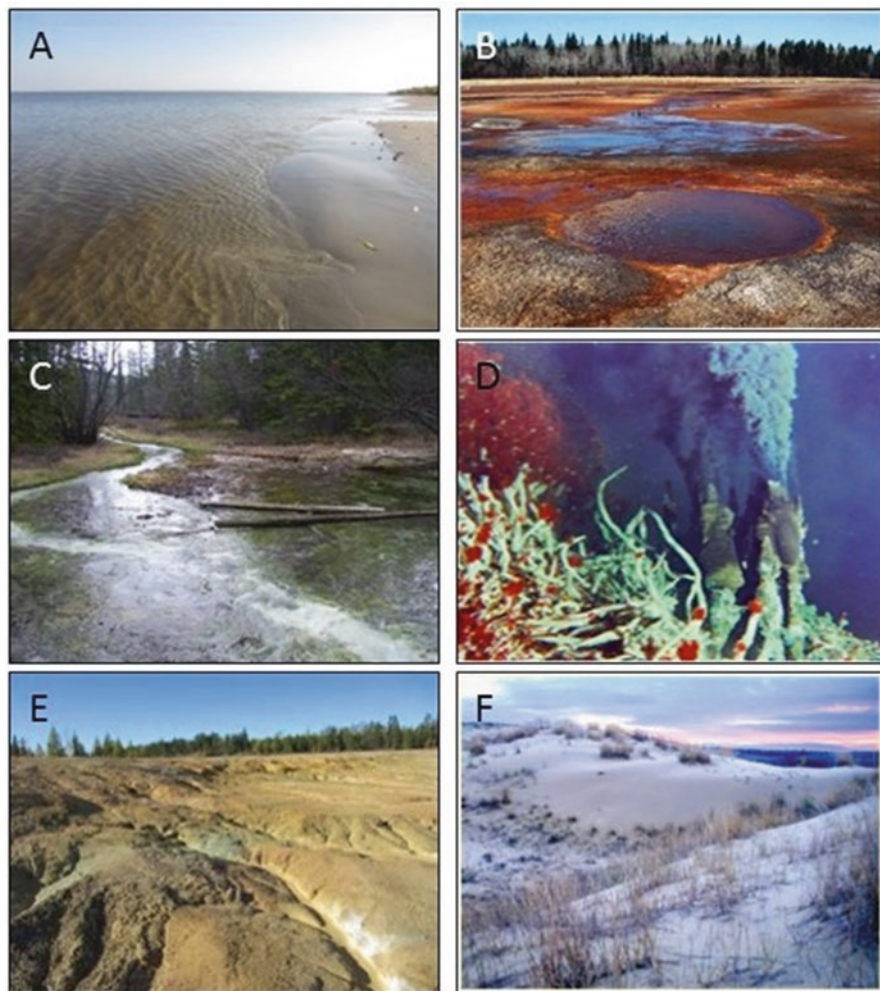


Fig. 1 Diversity of AAP habitats: (a) Lake Winnipeg, Manitoba, Canada; (b) East German Creek system, Northern Manitoba, Canada; (c) Sulfur Mountain hot springs, Banff, Alberta, Canada; (d) hydrothermal vent, Juan de Fuca Ridge, Pacific Ocean; (e) Central Mine, Nopiming Provincial Park, Canada; and (f) soil crusts, Manitoba, Canada

centers (RC) and light-harvesting (LH) complexes, resembling those of the purple non-sulfur bacteria (PNSB). However, while they do carry out anoxygenic photosynthesis in a similar manner as the PNSB, they differ in that it is not coupled to carbon fixation, as all AAP lack the key enzyme of the Calvin cycle, RUBISCO (Yurkov and Beatty 1998; Yurkov and Csotonyi 2009; Yurkov and Hughes 2013). Minimal levels of carbon fixation can occur through anapleurotic reactions in the TCA cycle, but it is not sufficient to support bacterial growth (Yurkov and Hughes 2013).

Phylogenetically, AAP can be most closely related to PNSB or to non-phototrophs among the α -, β -, and γ -proteobacteria. This raises questions into how the AAP evolved. When the atmosphere first became oxygenated 2.5 GYa ago through the action of oxygenic phototrophs such as cyanobacteria, it opened up new niches for life (Beatty 2005). At this point, did the AAP evolve from PNSB as selection pressure forced them to move into these newly formed habitats in contact with oxygen? Conversely, were the AAP originally aerobic heterotrophs that gained a photosynthetic gene cluster (PGC) through horizontal transfer from the PNSB? Or is it a combination of both those events? Have they remained closely related to the PNSB because they evolved from them by losing photosynthetic genes upon coming into contact with oxygen and then regained them later through horizontal gene transfer? There are speculations to support all these options (Yurkov and Csotonyi 2009; Yurkov and Hughes 2013), and in fact, the answer may be a combination of all proposals. This is only one of many mysteries still surrounding the AAP four decades post-discovery.

Despite intensive research, there are many unanswered questions about the AAP. Our chapter will explore the riddles, hypotheses, and new discoveries about the adaptations of the BChl *a* synthetic pathway in the presence of both light and oxygen, the role of carotenoids in light harvesting and photoprotection, the ecological significance of AAP in their natural habitats, as well as novel approaches to determine diversity and abundance in aquatic environments. It is primarily a review of scientific reports that appeared since 2013. For previous analyses of AAP, we recommend reading Yurkov and Beatty (1998), Rathgeber et al. (2004), Yurkov (2006), Yurkov and Csotonyi (2009), and Yurkov and Hughes (2013).

Diversity, Abundance, and Ecological Significance

Distribution and Enumeration

Investigations of AAP abundance and distribution have always been somewhat complicated. There is no defined medium that can be used to select specifically for AAP in culture-dependant approaches, as they grow best on complex carbon sources, allowing for a wide range of heterotrophs to thrive alongside them. Therefore, AAP must instead be identified among other colonies by their bright pigmentation, ranging from yellow, orange, and red to pink and purple. They should then be tested

spectrophotometrically for the presence of BChl *a* (Yurkov and Csotonyi 2009; Yurkov and Hughes 2013). Enumeration in such manners can result in an underestimation of abundance as a large percentage of microbes do not grow in a laboratory setting. Unfortunately, culture-independent sequencing methods are not accurate either, as there are no genes found to date that are exclusive to AAP (Yurkov and Hughes 2013). Some studies have tried to enumerate AAP in natural samples using detection of the *pufL* and *pufM* genes, which encode subunits of the RC (Yurkov and Hughes 2013). However, this can result in an overestimation of AAP abundance as the genes are too closely related to those of PNSB, making it impossible to distinguish between the two physiologically different groups with any certainty. If *pufLM* genes do not allow for an accurate study of abundance and distribution, are there any other genes restricted to the AAP that have not yet been identified? Full genome sequences of cultured strains may provide us with that answer.

Recently, the proposed solution to this conundrum has been to use infrared epifluorescence to detect BChl *a* in uncultured bacteria from both marine and freshwater habitats (Garcia-Chaves et al. 2016). While BChl *a* is the same light-harvesting pigment synthesized by PNSB, they are assumed to not be producing it in the presence of oxygen. Therefore, sample collection is limited to the aerated portion of the water column to ensure there is sufficient oxygen present and therefore any BChl *a* found must be from an aerobic bacterium (Mašín et al. 2012; Garcia-Chaves et al. 2016). This might be particularly effective in well-stratified lakes, where an aerated upper layer and an anaerobic bottom portion are separated by a sharp thermocline. Unfortunately, this method is also not completely accurate as several PNSB, for example, *Rhodobacter capsulatus*, can produce some BChl *a* in oxygenated environments (Hebermehl and Klug 1998).

Investigations in both marine and freshwater environments using infrared epifluorescence have revealed a great morphological diversity and abundance of AAP as well as their growth patterns. There was a clear seasonal variation with higher bacterial counts in summer and lower in winter (Cüperová et al. 2013; Ferrera et al. 2014; Fauteux et al. 2015) and also a clear negative correlation with distance from shore in marine habitats including the Delaware coast and estuary (Stegman et al. 2014), the Northeast Pacific and Arctic Ocean (Boeuf et al. 2013), and close to various islands in the Pacific Ocean (Ritchie and Johnson 2012), with AAP abundance decreasing as sampling sites became distanced from the shoreline. Apparently AAP make up a significant portion of the bacterial community in marine environments (10–14% of total bacteria in the Northeast Pacific and Arctic Oceans (Boeuf et al. 2013) and 3.5–7.9% in the Uwa Sea (Sato-Takabe et al. 2015)). The data agree with previous studies, showing that AAP compose roughly 10% of the bacterial community in the open ocean (Rathgeber et al. 2004; Yurkov and Csotonyi 2009; Yurkov and Hughes 2013). While most of the early distribution analysis of AAP was done in the open ocean, recently there has been increased interest in freshwater systems, where AAP represent as high as 29% of all prokaryotes in Austrian alpine lakes (Cüperová et al. 2013) and 2–12% in lakes throughout Germany, Finland, and Poland. Oligotrophic and mesotrophic lakes show higher presence of AAP over eutrophic lakes (Mašín et al. 2012), though earlier studies have suggested that enu-

meration can be even higher, up to 30 % in eutrophic habitats, such as freshwater hot temperature spring mats, which commonly play home to AAP (Yurkov and Beatty 1998; Yurkov and Hughes 2013).

Throughout all tested habitats, there is a broad taxonomic diversity of AAP species. However, in some environments, patterns are being recognized in the distribution of genera. For instance, in higher alpine lakes in Austria, almost all AAP belonged to the *Sphingomonadaceae* family. This could be because members of the family, such as the genus *Sphingomonas*, resist high levels of UV-B radiation (Cüperová et al. 2013). This may be a beneficial trait in regions where the water is very clear and the surrounding area has very little forest shade to protect the lake occupants from the damaging effects of intensive sunlight (Cüperová et al. 2013). Conversely, *Citromicrobium* relatives have been found to dominate in the aerated upper twilight zone in the Pacific Ocean, suggesting they may be able to use very dim light or specific wavelengths that can pass through the water column for photosynthesis, allowing them to occupy a specific niche that other AAP cannot (Zheng et al. 2015). Although this is one published speculation, in our opinion it has little merit, as all known AAP are able to grow indefinitely as heterotrophs without using light for photosynthesis. We propose that in this case, competition may be too high in illuminated spaces causing the *Citromicrobium* relatives to move into the upper twilight or dark zones, where they can effectively grow purely heterotrophically.

While some current methods allow observation of the patterns discussed above and achieve relatively accurate bacterial counts, culturing methods cannot be completely replaced. Only pure laboratory cultures of AAP allow us to study them in detail to define if our theoretical speculations are true and to discover novel physiological and biochemical traits that AAP possess.

Phylogenetic Conundrums

All AAP, like their PNSB counterparts, belong to the proteobacteria. The vast majority of taxonomically classified strains are part of the α -proteobacteria with one species, *Roseatales depolymerans*, belonging to the β -proteobacteria, and two species, *Congregibacter litoralis* and *Chromocurvus halotolerans*, to the γ -proteobacteria, though many culture-independent studies have shown that there are far more β - and γ -proteobacterial AAP present in nature, with the β -proteobacteria dominating freshwater environments and γ -proteobacteria being prominent in saline systems (Ritchie and Johnson 2012; Yurkov and Hughes 2013; Zheng et al. 2015; Lehours and Jeanthon 2015). One notable exception is found in the Northeast Pacific and Arctic Ocean, where β -proteobacteria were unexpectedly reported in high numbers when they are not usually observed in abundance in the open ocean (Boeuf et al. 2013).

Since our latest review (Yurkov and Hughes 2013), several novel genera and species have been described (Table 1): *Roseibacula alcaliphilum* isolated from Lake Doroninskoe, which is a meromictic soda lake in Siberia (Nuyanzina-Boldareva and

Table 1 Determinative characteristics of novel AAP described since 2012^a

Species	Habitat, site of isolation	Phylogenetic affiliation ^b	Color	Cell shape and size	In vivo BChl peaks
<i>Roseibacula alcaliphilum</i> ^c (Nuyanzina-Boldareva and Gorfenko 2014)	Lake Doroninskoe (meromictic soda lake) surface water	α -Proteobacteria, <i>Roseinatronobacter</i> and <i>Rhodobaca</i> (96%)	Pink	Oval, 0.5–1.0 × 1.5–1.7 μ m	810, 874 nm
<i>Citrimicrobium luteum</i> (Jung et al. 2014)	Gut of sea cucumber <i>Stichopus japonicus</i>	α -Proteobacteria, <i>Citromicrobium bathyomarinum</i> (98.4%)	Yellow	Cocci to ovoid rods, 0.8–1.0 × 1.0–1.5 μ m	ND ^d
<i>Humitalea rosea</i> (Margesin and Zhang 2013)	Petroleum hydrocarbon-contaminated soil, Italy	α -Proteobacteria, <i>Roseococcus</i> and <i>Rubritepida</i> (<92.8%)	Pale pink	Short rods, 0.9 × 1.4–1.7 μ m	764 nm (In vitro), In vivo ND
<i>Blastomonas aquatica</i> (Xiao et al. 2015)	Lake water on the Tibetan plateau	α -Proteobacteria, <i>Blastomonas natatoria</i> (99.0%)	Yellow to orange brown	Ovoid to rod	866 nm
<i>Gemmatimonas phototrophica</i> (Zeng et al. 2015)	Freshwater Swan Lake, Gobi Desert	Gemmatimonadetes, <i>Gemmatimonas aurantiaca</i> (96.1%)	Red	Rods, 0.3–0.5 × 1–6 μ m	819, 866 nm

^aNew species described before 2012 are summarized in Yurkov and Hughes (2013), Yurkov and Csotonyi (2009), Rathgeber et al. (2004), and Yurkov and Beatty (1998)

^bProteobacterial subclass, nearest 16S rRNA relative and phylogenetic distance to nearest relative

^cNames in bold indicate novel genera

^dND indicates not determined

Gorlenko 2014), and *Citrimicrobium luteum*, which is from the gut of a sea cucumber (Jung et al. 2014). Petroleum hydrocarbon-contaminated soil in Italy revealed the AAP *Humitalea rosea* (Margesin and Zhang 2013). *Blastomonas aquatica* was discovered in fresh to brackish water in Lakes Peng Co and Namtso on the Tibetan Plateau, China (Xiao et al. 2015). *Gemmatimonas phototrophica* is an interesting new species, which grows best under limited oxygen concentrations (Zeng et al. 2015). It is similar to *Roseicyclus mahoneyensis*, strain ML6, which is photosynthetically most active under microaerophilic conditions, though heterotrophic growth remains optimal aerobically (Rathgeber et al. 2012). Physiologically *G. phototrophica* groups better with AAP rather than PNSB, as it cannot grow in fully anaerobic conditions and produces BChl *a* in the dark. However, light does not inhibit its synthesis (Zeng et al. 2015), a trait which has also been observed in AAP strain EG13, isolated from a hypersaline spring system in Manitoba, Canada (Csotonyi et al. 2015). Another novel aspect is that it does not belong to the proteobacteria; it is part of the *Gemmatimonadetes* and not the *Proteobacteria*, which begs the question whether it should truly be recognized as AAP.

Recently, Koblížek considered a new definition of AAP (Koblížek 2015), which suggests to include all Bchl *a*-producing bacteria which, in laboratory conditions, grow primarily aerobically. This would allow the inclusion of the phototrophic methylotrophs and rhizobia as well as unusual species such as *G. phototrophica*. It was suggested that because they are aerobic BChl *a*-containing bacteria, they should be considered AAP despite other metabolic, taxonomical, and phylogenetic differences. Additionally, as more and more strains are undergoing full genome sequencing, it is being found that some species, which were presumed to be aerobic non-phototrophic heterotrophs, have all the necessary genes for fully operational photosynthetic pigment-protein complexes and photosynthetic electron transfer (Koblížek 2015). Having never produced BChl *a* and photosynthetic complexes in a controlled laboratory setting suggests that they are simply not being grown under appropriate conditions to express photosynthesis genes. One example is the obligately aerobic β -proteobacterium *Aquincola tertiarycarbonis*, strain L108, which was originally reported to not produce BChl *a* aerobically (Lechner et al. 2007). However, it does in fact synthesize photosynthetic protein complexes with bound BChl *a* when faced with a sudden decrease in organic carbon availability (Rohwerder et al. 2013). Hence, this species can now be re-categorized as an AAP. Such previously published misleading taxonomical descriptions open up the question of how many other AAP are masquerading as non-phototrophic heterotrophs in laboratory studies. Also, what growth conditions are best for inducing BChl *a* production in facultative phototrophs? Will patterns emerge or will every species behave differently? At this time we do not have the answers; however, studies on heterotrophs that have been sequenced to reveal the presence of a full PGC may help bring to light some solutions.

Morphology and Its Link to Ecological Significance

The AAP are morphologically highly diverse. They come in many forms: rods (both short and long), cocci, vibroid, almost cyclic as in *R. mahoneyensis* (Fig. 2a) (Rathgeber et al. 2005), and pleomorphic as found in *E. ramosum* (Fig. 2b), *Citromicrobium bathyomarimum* (Fig. 2c), and *Porphyrobacter meromictius* (Yurkov and Csotonyi 2009; Yurkov and Hughes 2013). They can form highly complex clusters that resemble corals or rosettes (Fig. 2d) (Yurkov and Hughes 2013). The only morphology that has not yet been observed is classic spirilloid, though studies in the Sargasso Sea indicate they may exist, but simply have not yet successfully been cultured in the laboratory (Sieracki et al. 2006).

Interestingly, on average, AAP cells in marine and freshwater habitats are larger than other members of their respective bacterial communities (Stegman et al. 2014; Fauteux et al. 2015; Garcia-Chaves et al. 2015; Sato-Takabe et al. 2015). In one study that analyzed samples from 43 lakes throughout Québec, Canada, the size of AAP cells was positively correlated with the amount of dissolved organic carbon (Fauteux et al. 2015). This size difference sets AAP apart from other heterotrophs in aquatic systems and has led to some interesting theories on their possible ecological significance and role.

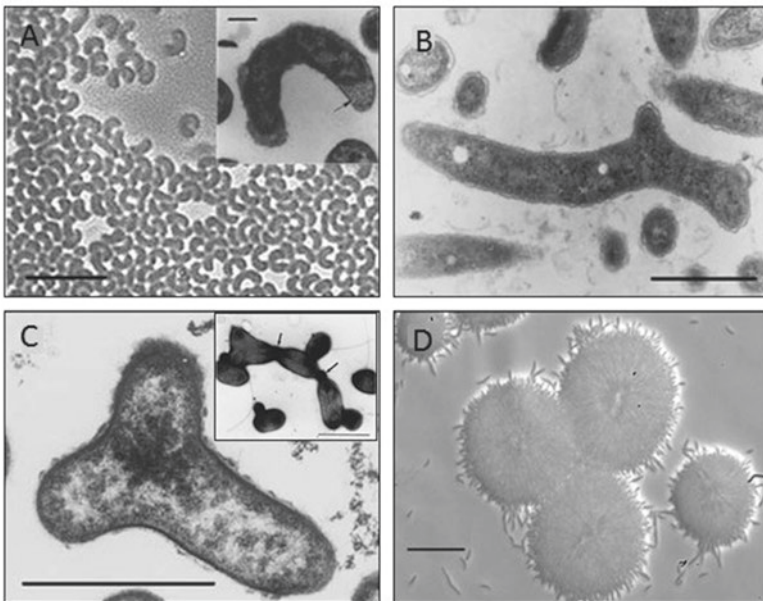


Fig. 2 Morphological variations among AAP. (a) *Roseicyclus mahoneyensis* with some almost cyclic cells (bar 5 μm , inset bar 0.25 μm); (b) branching *Erythromicrobium ramosum* (bar 1 μm); (c) *Citromicrobium bathyomarimum* Y-shaped cell (bar 1 μm), inset pleomorphic cells connected by membranous material (indicated by arrows, bar 1 μm); and (d) strain BL7 clusters (bar 2.5 μm)

As AAP are larger than the average non-phototrophic heterotrophic bacterium in communities, they are preferentially grazed upon by protists and zooplankton, placing them at the very bottom of the food chain in freshwater and marine ecosystems (Stegman et al. 2014; Garcia-Chaves et al. 2015; Mašín et al. 2012; Sato-Takabe et al. 2015). Zooplankton grazing in particular allows AAP energy to be passed to higher trophic levels (Garcia-Chaves et al. 2015). AAP also have a higher growth rate (1.5–3× higher) than some other heterotrophic bacteria (Stegman et al. 2014; Garcia-Chaves et al. 2015), possibly because they can profit by generating higher levels of energy more quickly due to their photosynthetic capacity. This allows the consumption of higher amounts of dissolved organic carbon at a faster rate before AAP are consumed by zooplankton, making a disproportionately large contribution to carbon cycling and total biomass considering their natural abundance (Stegman et al. 2014; Garcia-Chaves et al. 2015; Sato-Takabe et al. 2015).

When an organism at any trophic level is being grazed upon, it develops some defensive strategies (Mašín et al. 2012). It was proposed that AAP are no exception in this regard. In 27 studied lakes throughout Germany, Poland, and Finland, AAP were often found attached to particles that are too large to be consumed by zooplankton (Mašín et al. 2012). For now, it remains a speculation whether or not this is truly a defense mechanism. In our opinion, it is more likely AAP are simply attached to these particles as they are rich in organics, which serve as a good food source for bacteria rather than to be using them as an escape mechanism from preying zooplankton. In general, the ecological role of AAP in the majority of habitats is very poorly investigated. It still requires a lot of time and research effort to draw a clear image of their significance.

Carbon Utilization and Metabolic Pathways

Carbon Metabolic Pathways

It was established long ago that AAP are obligate heterotrophs as they lack the key enzyme RUBISCO of the Calvin cycle (Yurkov and Beatty 1998; Yurkov and Csotonyi 2009; Yurkov and Hughes 2013). Recent genome sequence data from a number of species including *Sandarakinorhabdus* sp. AAP62 (Zeng et al. 2013a), *Blastomonas* sp. AAP53 (Zeng et al. 2013b), *Porphyrobacter* sp. AAP82 (Li et al. 2013), and *Sphingomonas* sp. FukuSWIS1 (Salka et al. 2014) have only confirmed the absence of this cycle. While incapable of autotrophy, AAP, like many other heterotrophs, fix a minimal amount of CO₂ via anapleurotic reactions (Fig. 3) (Yurkov and Hughes 2013), though it is at insufficient levels to support growth.

What AAP do excel at is breaking down highly complex organics due to the activity of a broad range of proteins and enzymes. This makes them indispensable in carbon cycling in both saline and freshwater systems, as naturally available carbon is generally very complex both structurally and chemically for digestion by other microbes (Fauteux et al. 2015).

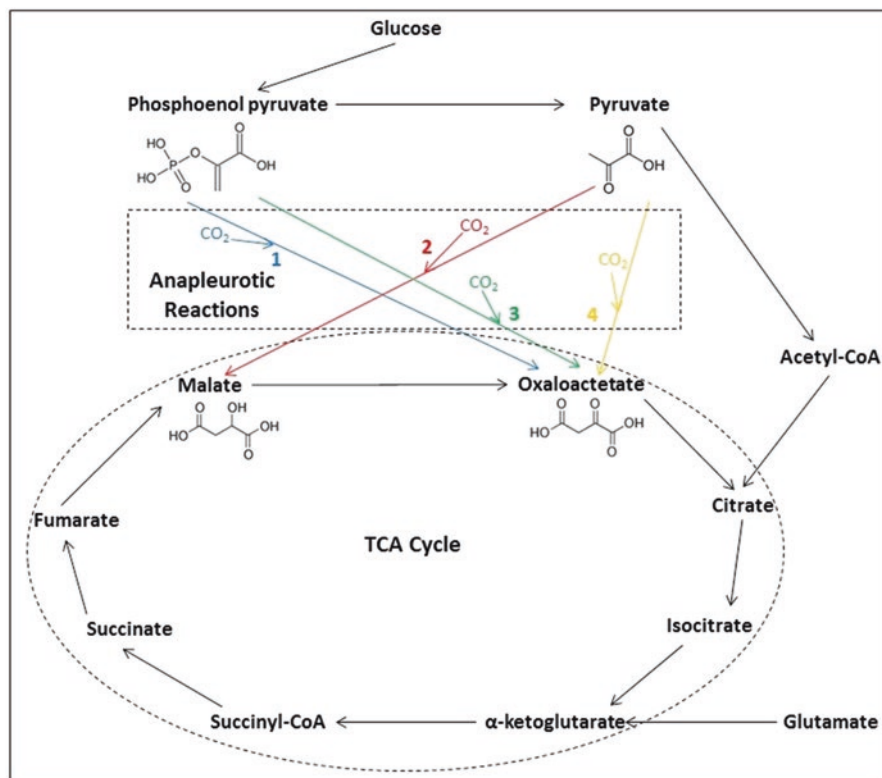


Fig. 3 Fixation of CO_2 through anapleurotic reactions encoded by the enzymes (1, *blue*) phosphoenolpyruvate carboxykinase, (2, *red*) malic enzyme, (3, *green*) phosphoenolpyruvate carboxylase, and (4, *yellow*) pyruvate carboxylase replenishes the TCA cycle. Glucose feeds into the TCA cycle through pyruvate and therefore anapleurotic reactions or acetyl-CoA. Glutamate replenishes the TCA cycle through α -ketoglutarate

Interestingly, when grown on defined medium, the carbon source can have an impact on levels of photosynthesis and anapleurotic reactions in *Erythrobacter* sp. NAP1 (Hauruseu and Koblížek 2012). Four commonly used compounds were tested: glutamate, pyruvate, acetate, and glucose. When provided with glutamate, growth was inhibited by high light intensities, though greater amounts of biomass were produced with a moderate amount of light. Additionally, almost none of the incorporated carbon came from anapleurotic reactions (1%). Conversely, light intensity made no difference when medium contained pyruvate instead of glutamate. However, 4% (dark) and up to 11% (light) of incorporated carbon originated from anapleurotic reactions (Hauruseu and Koblížek 2012). It seems to be species specific, as these values are lower than the 10–15% anapleurotic CO_2 fixation carried out by *R. denitrificans* (Hauruseu and Koblížek 2012). It was discovered long ago that photosynthetic activity allows AAP to increase their productivity (Yurkov and Gernerden 1993), and now it is confirmed that the organic carbon source also

makes a difference. For instance, light exposure allowed 30 % biomass increases, when grown in media containing pyruvate; however, photosynthesis in the presence of glucose resulted in 49.1 % increase (Hauruseu and Koblížek 2012).

It is possible that anapleurotic reactions are used by AAP simply to replenish the TCA cycle. Anapleurotic reactions use pyruvate or phosphoenolpyruvate as a substrate (Fig. 3); hence, more carbon fixation occurs in pyruvate- and glucose-grown cultures. Glutamate, however, can be easily converted to α -ketoglutarate to feed into the TCA cycle (Fig. 3) (Hauruseu and Koblížek 2012). It is also conceivable that this explanation ties into why light intensity makes a difference with glutamate and not with pyruvate. With glutamate, higher light intensities provide more energy than is required by the cell, which in the end causes downregulation of metabolism. However, anapleurotic reactions using pyruvate as a substrate require a large amount of energy, so the possibility of excess is avoided and metabolism is not downregulated (Hauruseu and Koblížek 2012).

Mechanisms of Toxic Heavy Metal(loid) Oxide Resistance and Reduction

As mentioned above, AAP are commonly enumerated in high numbers in extreme environments. While it remains uncertain how and why they have evolved mechanisms to allow growth in such a wide range of extremes, their capability to be very comfortable in the presence of toxic heavy metal(loid) oxides has been researched. These oxides include tellurite, tellurate, selenite, selenate, metavanadate, and orthovanadate (Yurkov and Csotonyi 2003). AAP show resistance to tellurite, for example, of up to 2000 $\mu\text{g/ml}$, a highly significant number considering that many bacterial species are killed by only 1 $\mu\text{g/ml}$ (Maltman and Yurkov 2015). How is such great resistance to metal(loid) oxides achieved?

One strategy of resistance by AAP is to use toxic compounds in metabolic processes or for energy production. Strains EG13 and EG8 are vanadiphilic, meaning vanadium oxides, which usually kill most life in very small concentrations, actually enhances the bacterial growth capabilities (Csotonyi et al. 2015). Similarly, *E. ursincola*, KR99, and *E. ramosum*, E5, increase biomass and ATP production when grown in the presence of tellurite (Maltman and Yurkov 2014, 2015). Alternately, instead of benefiting from the oxides, some species can simply tolerate them. For instance, *C. bathyomarinum*, JF1, experiences a lag phase when tellurite is added to the medium to adapt to the shock and then resumes normal biomass production (Maltman and Yurkov 2014). *R. thiosulfatophilus*, RB3; *E. ezovicum*, E1; *S. sibiricus*, RB 16-17; *E. hydrolyticum*, E4; and *Erythrobacter litoralis*, T4, all have decreased biomass and ATP levels in the presence of high concentrations of tellurite, suggesting it has a toxic effect on the cells (Maltman and Yurkov 2015).

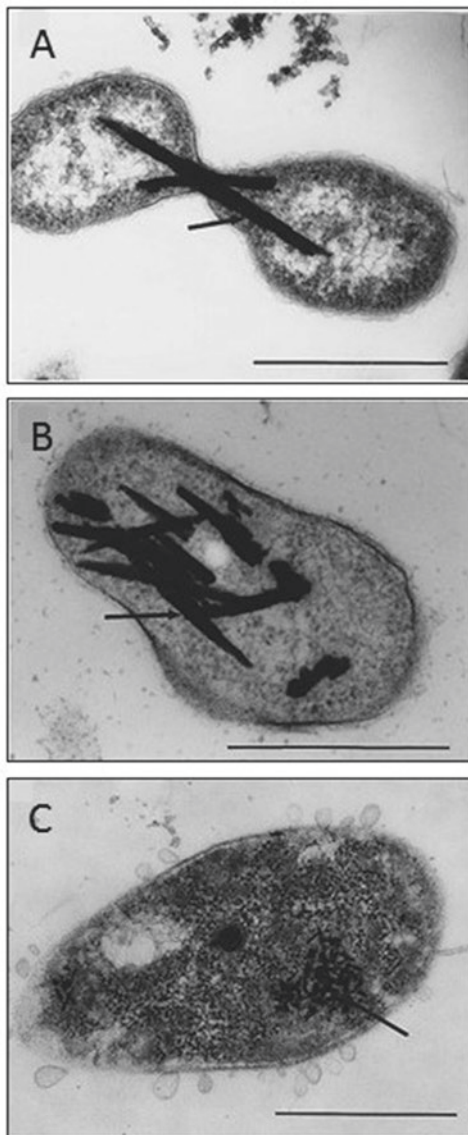
A search was also carried out for a tellurite-specific reductase in strains KR99, E5, RB3, E1, RB 16-17, E4, T4, and JF1. Cells were fractionated to determine in what compartment of the cell the tellurite reductase was localized. Additionally, protein

synthesis was halted with antibiotics before cells were introduced into the medium containing tellurite to check whether the enzyme was constitutively expressed or de novo synthesis was required. Reductase activity in strain E4 was only possible in fully intact cells and de novo protein synthesis was required. T4 and JF1 also had a requirement for de novo synthesis and needed an intact cytoplasmic membrane as was supported by activity in spheroplast lysate fractions (Maltman and Yurkov 2014). In the other tested strains, a constitutively expressed membrane-bound tellurite reductase was present, suggesting the function of a different enzyme (Maltman and Yurkov 2015). Future research will answer how other AAP resist such high levels of toxic compounds and in some cases have evolved to benefit from them.

One of the most common observations with high resistance to toxic metal(loid) oxide strains is that they reduce them to less toxic elemental forms, followed by color changes in the culture. Reduction of tellurite and tellurate to tellurium results in a black appearance, selenite and selenate conversion to selenium brings a red color, and vanadate being reduced to vanadium appears as bluish coloration (Yurkov and Csotonyi 2003; Maltman and Yurkov 2015). As oxides are reduced, the elements accumulate inside or are released outside the cells, as seen by electron microscopy of *E. ramosum* (Fig. 4a), *E. litoralis* (Fig. 4b), and *R. thiosulphatophilus* (Fig. 4c). In the future this could be an indispensable ability to remove toxic compounds for bioremediation in industrial processes, as well as offer a great potential for biometallurgy by collecting concentrated pure tellurium or other accumulated elements as they are quite rare in the biosphere.

Because AAP are so highly resistant to tellurite, in particular, the question was asked whether tellurite resistance is tied to photosynthesis. BChl *a* and carotenoids were monitored in *E. ramosum*, E5; *E. ursincola*, KR99; *C. bathyomarinum*, JF1; *E. litoralis*, T4; and *Erythrobacter* relative strain EG15 in the presence and absence of tellurite (Csotonyi et al. 2014). BChl *a* increased when cultures were grown with tellurite in strains KR99, JF1, T4, and EG15, indicating this pigment may play the role of an antioxidant, but decreased in strain E5, showing oxidative stress could be exceeding the cell's ability to cope. However, when carbon availability was decreased, BChl *a* levels increased in E5 despite the presence of tellurite (Csotonyi et al. 2014). The same pattern holds true for cellular carotenoids. They were increased in KR99, T4, and EG15 and decreased in E5, though under substrate limitation the carotenoids of E5 did increase. The only strain that broke the pattern is JF1, as its carotenoids decreased, while BChl *a* was augmented. Notably, in strain T4, zeaxanthin, spirilloxanthin, β -carotene, and erythroxyanthin sulfate were elevated, and bacteriorubixanthin levels declined (Csotonyi et al. 2014). Speculations into the above results suggested three hypotheses. First, tellurite may act on the promoters to induce transcription of the entire PGC, even if carotenoids are the only pigments involved in oxidative defense. Second, BChl *a* may act in conjunction with carotenoids as antioxidants. Third, resistance to tellurite may be quite energetically costly, causing the upregulation of the photosynthetic apparatus to supply the cells with as much additional energy as possible (Csotonyi et al. 2014). Ongoing experiments will help to tell which strategy is actually used by AAP cells.

Fig. 4 Electron microscopy showing intracellular accumulation of tellurium (indicated by arrows) as a product of tellurite reduction. (a) Tellurium crystals interfering with cell division in *E. ramosum*, (b) tellurium accumulated in *E. litoralis*, and (c) smaller tellurium crystals accumulated in *R. thiosulfatophilus*. Bars, 0.5 μ m



Photosynthetic Pigments and Their Significance

The Enigma of Aerobic Bacteriochlorophyll a Synthesis

The process of BChl *a* synthesis in the presence of both light and oxygen results in the formation of triplet BChl and singlet oxygen, which exerts high levels of oxidative stress on the cells (Yurkov and Hughes 2013). This is a barrier that all anoxygenic phototrophs have had to overcome. Some phototrophs, for instance, PNSB,

produce BChl *a* primarily under anaerobic conditions, decreasing the possibility of oxidative stress. Contrarily, AAP produce their photosynthetic pigments mainly in the dark, so they can then be used up during periods of light. Also AAP keep the number of photosynthetic units to a minimum to limit the toxic effect and have much higher levels of carotenoids to protect the cells from damage. One notable exception to the rule is strain EG13, which synthesizes BChl *a* with either illumination or not (Csotonyi et al. 2015). It has a very good strategy to deal with oxidative stress, possibly tied to its resistance to toxic vanadium oxides as described in section “Mechanisms of Toxic Heavy Metal(loid) Oxide Resistance and Reduction,” though continuing research is required for a better understanding of the exact mechanism (Csotonyi et al. 2015).

Genomic and proteomic studies have also been conducted to define the differences in BChl *a* synthesis in cells of AAP versus PNSB and chlorophyll *a* synthesis in oxygenic phototrophs such as cyanobacteria, as all synthetic pathways start similarly. One major difference is in the genes encoding the enzyme magnesium-protoporphyrin IX monomethylester cyclase (Boldareva-Nuianzina et al. 2013). There are two options possible. The gene *acsF*, which uses an O₂ molecule to catalyze the reaction, and the other, *bchE*, instead take an oxygen atom from H₂O (Fig. 5). The genome sequences of 53 phototrophic proteobacteria were compared. Purple sulfur bacteria contain only the *bchE* gene, PNSB all have both forms, and tested AAP all have *acsF*; however, several also have *bchE* (Boldareva-Nuianzina et al. 2013). This delivers a conundrum, as it was previously expected that anaerobic bacteria would have the oxygen-independent form and aerobic phototrophs, the oxygen-dependant type only. Speculatively, *acsF* was gained very early in evolution, as it has a highly conserved position within the PGC: in the *puh* operon in α - and β -proteobacteria and on the end of the PGC in γ -proteobacteria. Hypothetically, it was passed from cyanobacteria to PNSB

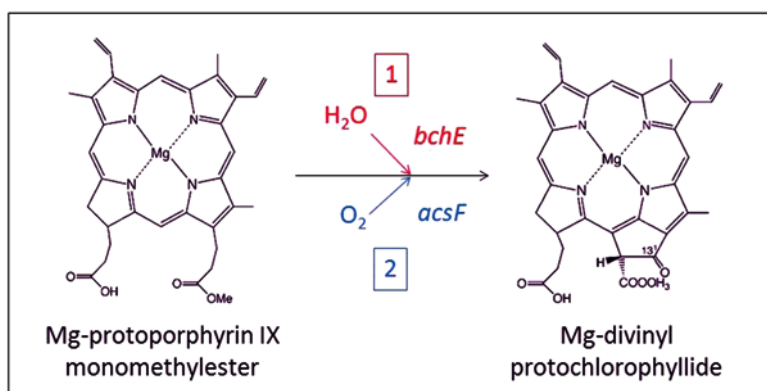


Fig. 5 Conversion of Mg-protoporphyrin IX monomethylester to Mg-divinyl protochlorophyllide using two different forms of the enzyme Mg-protoporphyrin IX monomethylester cyclase. The first form (1, red) is encoded by the gene *bchE* to use an oxygen atom from H₂O. The second (2, blue) is encoded by the gene *acsF* to use atmospheric O₂

through horizontal gene transfer, allowing PNSB to proliferate into oxygenated environments and initiate the evolutionary process to become AAP (Boldareva-Nuianzina et al. 2013). Likely, the gene was acquired very early in evolution, since a phylogenetic tree based on *acsF* sequences grouped all studied strains the same way as 16S rRNA gene sequences would. Therefore, the gene probably evolved in conjunction with the evolution of each species, rather than being a separate entity recently gained. This may not be true of *bchE*, which appears to be highly variable in position and sequence, as compared to the conserved 16S rRNA gene sequences (Boldareva-Nuianzina et al. 2013).

Another known step in BChl *a* synthesis is the conversion of protochlorophyllide to chlorophyllide by the enzyme protochlorophyllide reductase (Kaschner et al. 2014). There are two forms of this reductase: an oxygen-sensitive dark operating type, which is likely used by anoxygenic phototrophs, and an oxygen-insensitive light-dependant form, possibly employed by oxygenic phototrophs such as cyanobacteria and algae (Kaschner et al. 2014). However, the AAP *Dinoroseobacter shibae*, strain DFL12T, possesses both (Kaschner et al. 2014). Why would an anoxygenic phototroph use an enzyme for BChl *a* synthesis that requires both light and oxygen in conjunction as synthesis of the pigment in this way results in oxidative stress? This is just another unanswered question. It seems that as we explore deeper into AAP BChl *a* synthesis, we just come up with more questions, riddles, and mysteries.

Carotenoids and Their Role in AAP

In comparison to PNSB, AAP have a very high cellular presence of carotenoids, resulting in the vibrant colors of their cultures, such as red, orange, yellow, pink, and purple (Rathgeber et al. 2004; Yurkov and Csotonyi 2009; Yurkov and Hughes 2013). The roles of cellular carotenoids are still debatable, although two are proposed. The first considers their use as accessory light-harvesting pigments in photosynthesis, which may allow the cells to harvest light in a wider range of wavelengths, maximizing the efficiency of photosynthetic energy generation. The second suggests that carotenoids are protecting cells against the damaging effects of light, permitting BChl *a* synthesis aerobically. Realistically, the answer might be a combination of both, with some carotenoids bound to the photosynthetic RC to aid in light harvesting and others acting as protectors against the oxidative stress.

One clear example of an organism that likely uses carotenoids in light harvesting is *Roseobacter*, strain OBYS0001, which has spheroidenone as its dominant pigment (Sato-Takabe et al. 2012, 2014). Higher levels of spheroidenone were produced under substrate-deficient conditions. Apparently, pigments may provide some benefit to starving cells. Combined with the knowledge that this carotenoid absorbs green light and that under substrate deficiency the photochemical efficiency was increased compared to in substrate-replete conditions, the cells may be collecting green light to increase their photosynthetic energy generation. Hence

cells can survive, when other heterotrophs would perish (Sato-Takabe et al. 2012). Energy transfer from spheroidenone to BChl *a* was also confirmed by Šlouf et al. (2013) in *R. denitrificans*.

Zeaxanthin, one of the major pigments in *Erythrobacter* sp. NAP1, is not being used for energy transfer (Šlouf et al. 2013), suggesting that its main role is in protection against the aggressive triplet BChl *a* and singlet oxygen (Šlouf et al. 2013). However, the organism also has significant levels of bacteriorubixanthinal, which was shown to harvest light energy and pass it to BChl *a* (Šlouf et al. 2013). A similar composition of carotenoids was previously reported in *E. longus* and *E. litoralis*, though *Erythromicrobium* species such as *E. ramosum* have zeaxanthin only as a minor component of their carotenoid complement with high levels of bacteriorubixanthinal, erythroanthin sulfate, and β -carotenes (Yurkov et al. 1993b; Yurkov and Beatty 1998). *R. thiosulfatophilus* is different as 95 % of its carotenoid complement is the C₃₀ carotenodioate and its diglucosylester (Yurkov et al. 1993a; Yurkov and Beatty 1998), both of which participate in light harvesting.

One AAP species can have numerous different carotenoids, in some cases up to 20 or more, with each one presumably playing a different role. Whether all species have carotenoids that aid in light harvesting, or if the majority are used for protection, or if there are other metabolic functions to carry out is still unresolved. The only way to answer is to continue culture-dependent studies of carotenoids in AAP.

Photosynthetic Apparatus and Electron Transport Chain

New Discoveries on Reaction Centers and Light-Harvesting Complexes

The membrane-bound pigment-protein complexes making up the photosynthetic apparatus in AAP are almost identical to those of the PNSB. BChl *a* is the primary light-harvesting pigment bound into a RC and LH1 complex, with some species also employing a peripheral LH2. The RC can be observed spectrophotometrically by producing absorption peaks at 800 nm and 860–870 nm due to the incorporated BChl *a* as well as a peak at 750–760 nm of bacteriopheophytin (BPheo), a BChl precursor, also bound to the RC proteins. It is a complex composed of three protein subunits (L, H, and M) with four bound BChl *a* molecules, two BPheo, two ubiquinones, a nonheme iron, and carotenoids (Yurkov and Beatty 1998; Yurkov and Csotonyi 2009; Yurkov and Hughes 2013). There can be anywhere between 140 and 1800 RC per cell, a full order of magnitude less than what is produced by the PNSB (Selyanin et al. 2015). The small number of photosynthetic units is seemingly to keep the oxidative stress in the cells (caused by the formation of triplet BChl *a* and singlet oxygen) to a minimum, while still taking advantage of as much of the energy generation photosynthesis allows for.

The LH1 complex can also be observed spectrophotometrically typically as an absorption peak at 870 nm, though this can be somewhat shifted: at 879 nm in *C.*

halotolerans and *CharonOMICROBIUM ambiphototrophicum* or as far as 855 nm in *R. thiosulfatophilus* (Yurkov and Csotonyi 2009; Yurkov and Hughes 2013). These shifts are caused by varying protein environments of BChl *a*. Structurally, the LH1 complex is a ring of 16 heterodimeric alpha and beta subunits with approximately 30 BChl *a* molecules and bound photosynthetic carotenoids (Yurkov and Beatty 1998; Tang et al. 2010).

The LH2 complex is found in only some AAP species and, when present, allows for more efficient photosynthetic activity. It has a similar ring structure as the LH1, but is composed of only 8–9 heterodimeric alpha/beta subunits (Tang et al. 2010). Spectrophotometrically two peaks are present: one at around 805 nm and the other at 850 nm. However, there can also be shifts in the peaks, indicating differences in the protein settings may exist from species to species. Absorbance maxima have been seen, for instance, at 832 nm in *E. ramosum* or 835 nm in *Porphyrobacter dokdonensis* (Yurkov and Hughes 2013). Additionally, in rare cases such as in *R. mahoneyensis*, the LH2 complex has a monomodal peak, absorbing at 805 nm, instead of the typical dual peaks (Rathgeber et al. 2005). This unusual monomodal complex has only been found in two other genera, *Roseobacter* and *Rubrimonas* (Shiba 1991; Suzuki et al. 1999).

The genes encoding the LH1 complex are located alongside those for the RC L and M subunits in the *puf* operon, while the RC H subunit is placed in the *puh* operon. Alpha and beta portions of the LH2 complex are placed in the *puc* operon (Zheng et al. 2011; Yurkov and Hughes 2013). Encoding the LH2 on a separate operon from the RC-LH1 complex permits independent gene regulation. This means that cells may turn on or off the genes for the LH2 without entirely eliminating photosynthetic activity as has been observed in *Citromicrobium litoralis* (Spring et al. 2009).

Recent studies have shown that exposure to light can decrease cellular respiration by about 25%. Obviously, AAP can replace oxidative phosphorylation with photophosphorylation to some extent (Hauruseu and Koblížek 2012). Also, light intensity can have an effect on the size of the RC-LH1 complex. *Roseobacter litoralis* under high light intensity had more RC-LH1 complexes than under low light. The complexes were smaller in cells grown in the light (39 ± 3 BChl *a* molecules) compared to grown in the dark (115 ± 30 BChl *a* molecules) (Selyanin et al. 2015). It's likely that the cultures grown under very low illumination had larger antennae to maximize light harvesting, and when grown under intensive light, they are smaller to reduce damage to the RC and cells from the high illumination (Selyanin et al. 2015). It would be interesting to study if this pattern holds true for other AAP species, both marine and freshwater.

The Photosynthetic Electron Transport Chain

Once light energy has been harvested by BChl *a*, it has to be funneled through the LH complexes into the RC, so the light energy can be converted into chemical energy (ATP) for further use in different metabolic processes. This is where the

cyclic photosynthetic electron transport chain comes in. Once BChl *a* is excited, the energy is passed to the primary electron acceptor, which is a quinone (Q_A). It is then passed to cytochromes (cyt), first the membrane-bound *cytbc*₁ complex and then the *cyt*_c, which is either soluble in the periplasmic space or tightly bound to the RC. The electron is then cycled back to the beginning of the electron transport chain (Yurkov and Hughes 2013).

While the chain described above is highly similar in AAP and PNSB, there are a couple of key differences. The first, and possibly most important, is that in AAP the Q_A redox midpoint potential is quite high (in the range of +5 to +150 mV, depending on the species) (Yurkov and Beatty 1998; Rathgeber et al. 2012) compared to the quinone in PNSB, which is always negative. This may be among the major reasons why AAP can only carry out photosynthesis in the presence of oxygen. The Q_A would be over-reduced under anaerobic conditions, making it incapable of accepting more electrons, so the cyclic electron transport comes to a grinding halt (Rathgeber et al. 2004; Yurkov and Csotonyi 2009; Yurkov and Hughes 2013). Second, PNSB have an alternative quinol oxidase pathway to keep the Q_A in the appropriate redox state (Yurkov and Csotonyi 2009; Yurkov and Hughes 2013). If the AAP had such pathways, they could possibly photosynthesize anaerobically. There are species, *R. mahoneyensis*, strain ML6, and *C. ambiphototrophicum*, EG17, in particular, that developed ways around this problem as they are AAP/PNSB intermediates (Rathgeber et al. 2005, 2012; Csotonyi et al. 2011). ML6 is most active photosynthetically under microaerophilic conditions, and EG17 produces the same photosynthetic pigment-protein complexes in both the presence and absence of oxygen. Future experimentation on intermediate species may help to find the principle difference between AAP and PNSB photosynthesis.

Further Riddles and Concluding Remarks

While investigations of AAP have made great leaps in the past several years, there is still so much we do not understand, and every discovery we make delivers new questions that have yet to be answered. We still discuss without much certainty whether AAP evolved from PNSB or the heterotrophs, as well as how and why they evolved coping strategies for so many extreme conditions. Based on the wide range of recently discovered primarily aerobic bacteria that are capable of BChl *a* production and oxygen-dependent anoxygenic photosynthesis, it might be the right time for a more inclusive definition of the term AAP. Modern ecological studies are at last revealing some potential roles AAP may play in aquatic systems; however, we must continue our experimentations to make more definitive remarks. Additionally, we still have not conclusively agreed on what sets the AAP photosynthetic electron transport chain and BChl *a* synthetic pathways apart from those of the PNSB, which allows them to function aerobically and restricts anaerobic flow of photosynthetic reactions. These are only a few of the many mysteries left to solve. Continued research is necessary to understand such an abundant, diverse, and fascinating group of bacteria.

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