Beyond the Calvin Cycle: Autotrophic Carbon Fixation in the Ocean

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Abstract

Organisms capable of autotrophic metabolism assimilate inorganic carbon into organic carbon. They form an integral part of ecosystems by making an otherwise unavailable form of carbon available to other organisms, a central component of the global carbon cycle. For many years, the doctrine prevailed that the Calvin-Benson-Bassham (CBB) cycle is the only biochemical autotrophic CO_2 fixation pathway of significance in the ocean. However, ecological, biochemical, and genomic studies carried out over the last decade have not only elucidated new pathways but also shown that autotrophic carbon fixation via pathways other than the CBB cycle can be significant. This has ramifications for our understanding of the carbon cycle and energy flow in the ocean. Here, we review the recent discoveries in the field of autotrophic carbon fixation, including the biochemistry and evolution of the different pathways, as well as their ecological relevance in various oceanic ecosystems.

INTRODUCTION

Autotrophic organisms have the ability to build all cell material solely from inorganic carbon. This makes autotrophic processes a crucial component of the global carbon cycle by providing the organic carbon used by heterotrophic organisms, which oxidize organic carbon back to inorganic carbon, completing the carbon cycle. The balance between autotrophy and heterotrophy is a key factor regulating CO_2 and O_2 concentrations in the atmosphere, and it also affects the overall redox balance of Earth. Although the standing stock of primary producers is much smaller in the ocean compared with the land, approximately half of the global primary production occurs in the ocean due to a much higher turnover of biomass (Field et al. 1998). In the ocean, most primary production occurs in the photic zone by cyanobacteria (e.g., *Prochlorococcus*, *Synechococcus*) and eukaryotic algae (e.g., diatoms and coccolithophorids), using the Calvin-Benson-Bassham (CBB) cycle for carbon fixation and oxygenic photosynthesis to conserve energy (Raven 2009, Scanlan et al. 2009, Bowler et al. 2010). This makes the CBB cycle the most significant carbon fixation pathway on today's Earth, and numerous reviews have summarized our understanding of this cycle and the importance of oxygenic photosynthesis for primary production on our planet (Shively et al. 1998, Tabita 2004, Tabita et al. 2007, Falkowski et al. 2008, Raven 2009).

Although other so-called alternative carbon fixation pathways have been known to exist for a long time, recognition of their relative contribution to local organic matter production and an appreciation of the ecological role of the organisms using these pathways have occurred only this past decade (Raven 2009). Presently, five CO_2 fixation pathways are known in addition to the CBB cycle: the reductive tricarboxylic acid (rTCA) cycle; the reductive acetyl-CoA, or Wood-Ljungdahl (WL) pathway; and the 3-hydroxypropionate (3-HP) bicycle; as well as the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) and dicarboxylate/4-hydroxybutyrate (DC/4-HB) cycles (Ljungdahl 1986, Buchanan & Arnon 1990, Berg et al. 2007, Huber et al. 2008, Zarzycki et al. 2009). This review focuses on microorganisms that are known to utilize alternative carbon fixation pathways and that play important roles in a range of oceanic ecosystems.

Microorganisms are usually grouped according to the metabolic strategies for obtaining carbon and energy. Possible energy sources are either sunlight or reduced chemical compounds, which drive phototrophic or chemotrophic lifestyles, respectively. Chemotrophic organisms are further subdivided into lithotrophs or organotrophs, depending on whether their source of chemical energy is derived from inorganic or organic compounds, respectively. Because bacteria and archaea are known for their versatile metabolism, mixotrophy is a widespread phenomenon, especially in aquatic environments (Eiler 2006; Moran & Miller 2007 and references therein). Mixotrophic organisms use several metabolic strategies simultaneously (e.g., incorporating organic carbon into cellular material using light and/or inorganic chemical energy sources), or they can switch between different strategies. In the context of the present review, we will discuss carbon mixotrophs that assimilate organic compounds in addition to the fixation of CO_2. Alternative CO_2 fixation pathways, e.g., the rTCA cycle, the 3-HP bicycle, or the 3-HP/4-HB cycle, may facilitate the assimilation of simple organic substances, e.g., acetate, succinate, or propionate, as these substances are intermediates of these pathways. Thus, carbon mixotrophs using such pathways have a competitive advantage over obligate autotrophs or heterotrophs (see the section Case Study 2: Autotrophic Processes in the Pelagic Realm, below).

Alternative carbon fixation pathways are used predominantly by chemolithoautotrophic bacteria and archaea. Chemolithoautotrophs, anaerobes in particular, are likely to have been among the first types of organisms on Earth (Martin et al. 2008, Berg et al. 2010a). They serve critical functions for ecosystems by providing fixed carbon and by closing element cycles (e.g., nitrogen, sulfur), because they catalyze inorganic redox reactions to obtain energy and reducing equivalents for the formation of organic molecules from CO_2, using a variety of inorganic compounds as

electron donors (e.g., S^{2-}, NH_4^+, H_2) and electron acceptors (e.g., O_2, CO_2, SO_4^{2-}, S°, NO_3^-). The energy yield depends on the thermodynamics of the redox couple as well as on the biochemistry of the pathways utilized (Fuchs 1989, McCollom & Amend 2005, Berg et al. 2010a). Thermodynamically, more energy is required to reduce inorganic carbon to organic carbon in aerobic, oxidized environments compared with anaerobic, reducing habitats (McCollom & Amend 2005). In line with this, CO_2 fixation pathways that harbor oxygen-sensitive enzymes and thus are used by anaerobic or microaerophilic microorganisms (reductive acetyl-CoA pathway, rTCA cycle, DC/4-HB cycle) require significantly less energy for synthesizing a three-carbon unit from CO_2 compared with the pathways that also function under fully aerobic conditions (CBB cycle, 3-HP bicycle, 3-HP/4-HB cycle; i.e., 1–5 ATPs for the synthesis of pyruvate versus 7–9 ATPs, respectively) (Fuchs 1989, Berg et al. 2010a; see **Supplemental Table;** follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**). Consequently, most aerobic chemolithoautotrophs use the oxygen-tolerant, but energy-demanding, CBB cycle, whereas anaerobic chemolithoautotrophs that are usually energy-limited utilize more energy-efficient, yet oxygen-sensitive carbon fixation pathways.

Supplemental Material

BIOCHEMISTRY AND GENOMICS OF CO_2 FIXATION PATHWAYS

In the following section, we describe the biochemical outlines of the different autotrophic carbon fixation pathways. In addition to the biochemistry of the pathways, we present genomic data, the phylogentic distribution of the pathways (**Figure 1**), as well as some historical aspects.

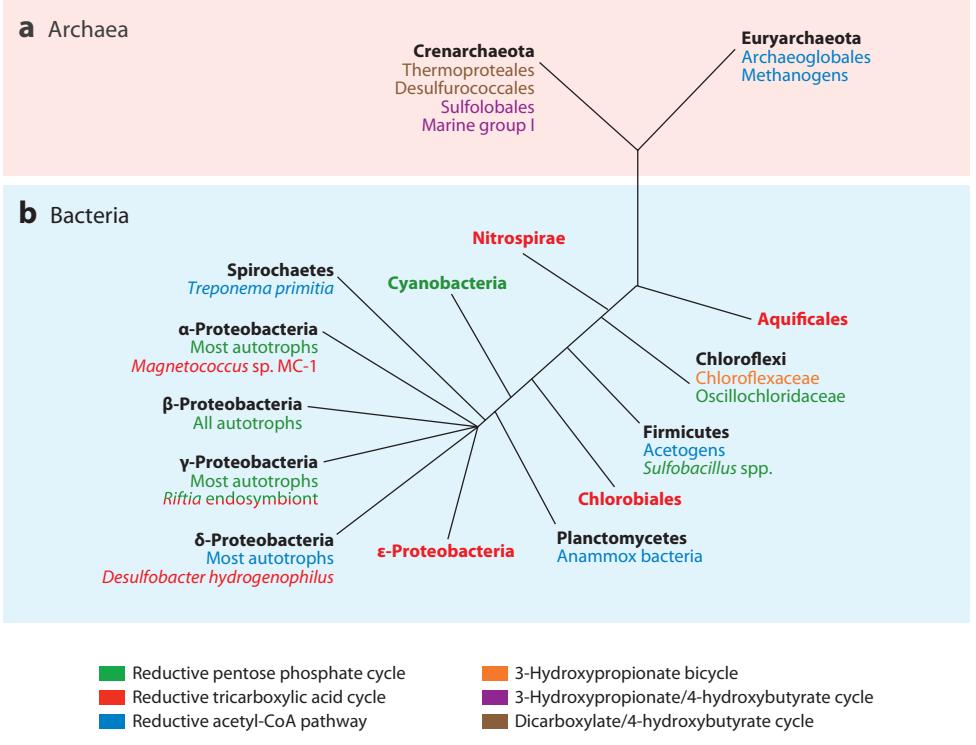


Figure 1

Schematic phylogenetic tree depicting the distribution of the six carbon fixation pathways among major phylogenetic lineages in (*a*) archaea and (*b*) bacteria. Adapted by permission from Macmillan Publishers Ltd.: *Nature*, 2005, 437:343–48, copyright 2005.

Calvin-Benson-Bassham Cycle

The complete CBB cycle (or reductive pentose phosphate cycle) was described in 1954 by the research group of Melvin Calvin (Bassham et al. 1954), and it was thought for quite some time that the CBB cycle might be the only carbon fixation pathway on Earth. The characteristic enzyme involved in the cycle is ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), which catalyzes the primary carboxylation of ribulose 1,5-bisphosphate, yielding two molecules of 3-phosphoglycerate. First described in 1957 (Quayle et al. 1957), RubisCO was and still is intensively studied. Presently, four different types of RubisCO proteins are known (forms I–IV). However, only forms I, II, and III catalyze the carboxylation reaction, whereas form IV—the RubisCO-like protein—is involved in other reactions (Tabita et al. 2007). Nevertheless, phylogenetic analyses support a common origin of all types of RubisCO that predates the split between bacteria and archaea (Tabita et al. 2007, Tabita et al. 2008). Apart from RubisCO, the enzyme phosphoribulokinase is essential for a functional CBB cycle. The CBB cycle probably evolved in cyanobacteria, and it is the only carbon fixation pathway operating in eukaryotes (algae and plants) as a result of the endosymbiotic acquisition of a cyanobacterium that evolved into the chloroplasts. Overall, the phylogenetic diversity of bacterial groups using the CBB cycle is rather limited (**Figure 1**). Besides cyanobacteria, the CBB cycle occurs in photo- and (aerobic) chemoautotrophic Alpha-, Beta-, and Gammaproteobacteria. In addition, some Gram-positives (*Sulfobacillus* spp., Firmicutes) and members of the Oscillochloridaceae (Chloroflexi) use this cycle (Caldwell et al. 2007, Ivanovsky et al. 1999).

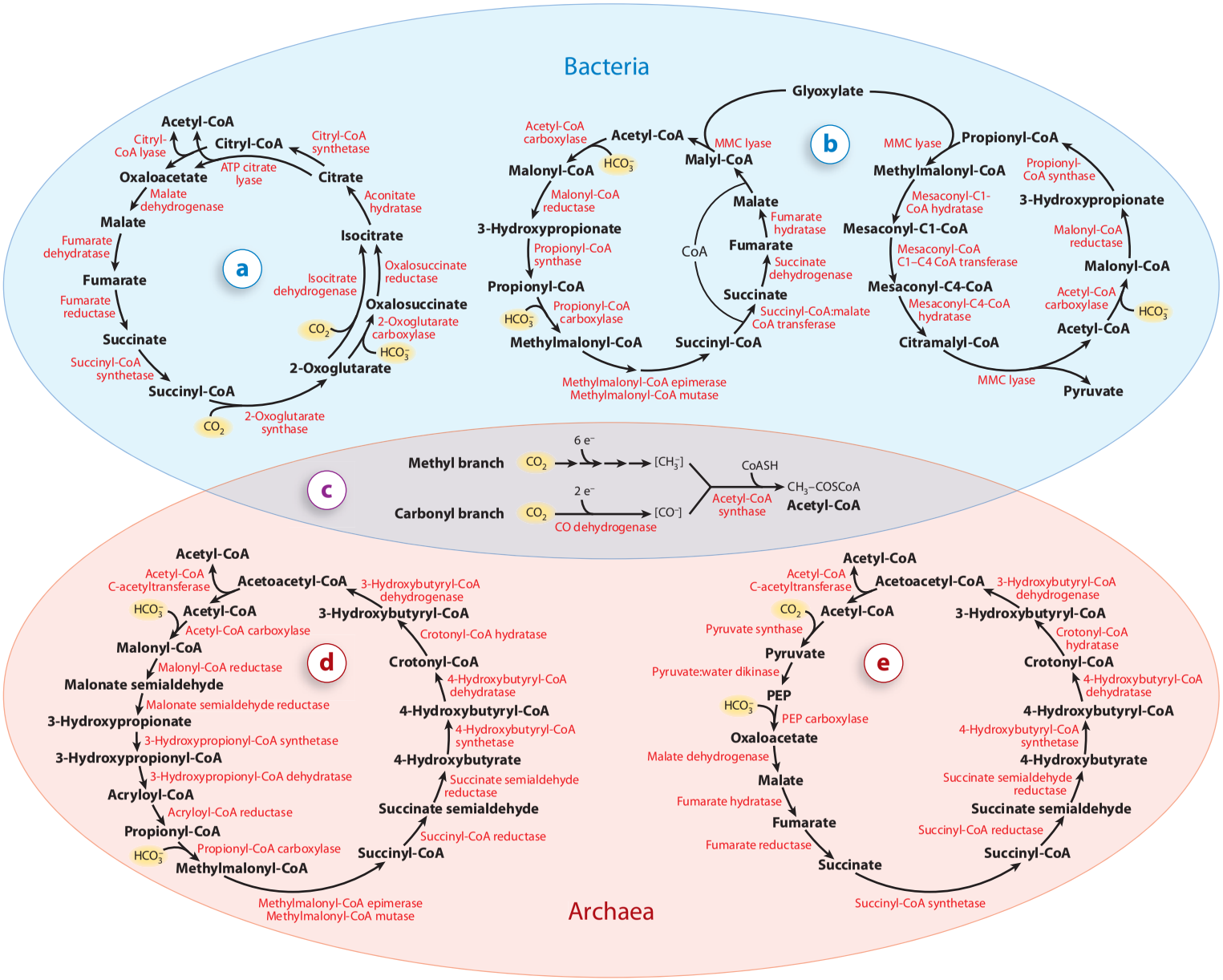
Reductive Tricarboxylic Acid Cycle

The rTCA cycle was first proposed in 1966 to act as a CO_2 fixation pathway in *Chlorobium thiosulfatophilum* (now *Chlorobium limicola*) (Evans et al. 1966). However, it took until 1980 for this pathway to become generally accepted (Buchanan & Arnon 1990). Over the last 10 years, the understanding of the biochemistry, evolution, and ecology of the rTCA cycle has increased considerably. Model organisms for elucidating the biochemistry of the pathway included the green sulfur bacteria *C. limicola* and *Chlorobaculum tepidum*, as well as *Hydrogenobacter thermophilus* (Aquificales) (reviewed in Buchanan & Arnon 1990 and Aoshima 2007).

The rTCA cycle is essentially a reversal of the oxidative TCA cycle, or Krebs cycle (**Figure 2*a***). Whereas the oxidative TCA cycle is used in heterotrophic organisms to oxidize acetyl-CoA to CO_2, thereby generating reducing power for the synthesis of adenosine triphosphate (ATP), the rTCA cycle can be used for the reverse process, the biosynthesis of acetyl-CoA from two molecules of CO_2. Most enzymes (malate dehydrogenase, fumarate hydratase, succinyl-CoA synthetase, isocitrate dehydrogenase, aconitate hydratase) can be used in both variants of the cycle because they catalyze fully reversible reactions. Unique enzymes of the reductive TCA cycle are fumarate reductase, 2-oxoglutarate synthase (2-oxoglutarate:ferredoxin oxidoreductase), and the citrate cleaving enzymes. Two carboxylation reactions are involved: the reductive carboxylation of succinyl-CoA to 2-oxoglutarate, catalyzed by 2-oxoglutarate synthase, and the reductive

Figure 2

Alternative pathways of autotrophic CO_2 fixation: (*a*) reductive tricarboxylic acid cycle, (*b*) 3-hydroxypropionate bicycle, (*c*) reductive acetyl-CoA pathway, (*d*) 3-hydroxypropionate/4-hydroxybutyrate cycle, and (*e*) dicarboxylate/4-hydroxybutyrate cycle. The biochemical reactions involved, as well as the enzymes (*red*) catalyzing the reactions, are depicted. Note that the reductive acetyl-CoA pathway is so far the only known CO_2 fixation pathway used by bacteria as well as archaea.



carboxylation of 2-oxoglutarate to isocitrate. The latter reaction can be accomplished either by isocitrate dehydrogenase, as shown for *C. limicola* (Kanao et al. 2002), or by the enzymes 2-oxoglutarate carboxylase and oxalosuccinate reductase, with oxalosuccinate as a free intermediate, as described for *H. thermophilus* (Aoshima 2007). The ATP-dependent cleavage of citrate to acetyl-CoA and oxaloacetate can be considered the key reaction of the rTCA cycle. This complex reaction can be achieved either by ATP citrate lyase or by the combined action of citryl-CoA synthetase and citryl-CoA lyase (see the section Citrate Cleavage, below). In order to get pyruvate from acetyl-CoA, another carboxylation reaction, catalyzed by pyruvate synthase, is required (Evans et al. 1966).

Phylogenetic distribution. The rTCA cycle is present in quite diverse groups of bacteria; however, due to the oxygen sensitivity of the enzymes 2-oxoglutarate and pyruvate synthase, the cycle appears to be restricted to anaerobic or microaerophilic bacteria (**Figure 1**). Enzymatic as well as genomic data suggest that all members of the obligate photoautotrophic and anaerobic green sulfur bacteria (Chlorobiales) use the rTCA cycle for carbon fixation (Wahlund & Tabita 1997, Buchanan & Arnon 1990, Kanao et al. 2002). Other bacterial groups in which all autotrophic members are believed to use the rTCA cycle are the Aquificales and the Epsilonproteobacteria. Enzymatic evidence has been obtained for several strains of Aquificales and Epsilonproteobacteria (Shiba et al. 1985; Beh et al. 1993; Hügler et al. 2005, 2007; Takai et al. 2005), and the sequenced genomes also provide evidence for the comprehensive use of this pathway in these groups (Deckert et al. 1998, Nakagawa et al. 2007, Sievert et al. 2008b, Reysenbach et al. 2009).

The operation of the rTCA cycle has also been reported for members of other bacterial groups, including the magnetotactic Alphaproteobacterium *Magnetococcus* sp. MC-1 (Williams et al. 2006), the Deltaproteobacterium *Desulfobacter hydrogenophilus* (Schauder et al. 1987), and "*Candidatus* Endoriftia persephone," the gammaproteobacterial endosymbiont of the giant tubeworm *Riftia pachyptila* (Markert et al. 2007). In the case of the *Riftia* symbiont, the rTCA cycle seems to be used in addition to the CBB cycle, making it the first bacterium that most likely expresses two different carbon fixation pathways simultaneously (Markert et al. 2007). In addition, genome analyses of acidophilic iron-oxidizing *Leptospirillum* spp. strongly suggest the operation of the rTCA cycle in autotrophic members of Nitrospirae (Levican et al. 2008). This group also contains the nitrite-oxidizing genus *Nitrospira*, and recent genomic and isotopic data suggest the usage of the rTCA cycle in "*Candidatus* Nitrospira defluvii" as well (Lücker et al. 2010). Initially, it was thought that the rTCA cycle also operates in certain archaea (e.g., *Thermoproteus neutrophilus* or *Pyrobaculum islandicum*; Beh et al. 1993, Hügler et al. 2003a); however, recent data suggest that they use the DC/4-HB cycle for carbon fixation (Ramos-Vera et al. 2009).

Citrate cleavage. The key reaction of the rTCA cycle is the ATP-dependent cleavage of citrate into acetyl-CoA and oxaloacetate. As mentioned above, this quite complex reaction can be achieved with different enzymes, which have been studied in some detail. In *H. thermophilus*, citryl-CoA synthetase (CCS) catalyzes the ATP- and CoA-dependent activation of citrate to citryl-CoA (Aoshima 2007) (**Figure 3**). This enzyme is composed of two subunits that have sequence similarities to succinyl-CoA synthetase. Thus, CCS may have evolved from succinyl-CoA synthetase after a gene duplication event (Aoshima 2007). Subsequently, citryl-CoA lyase (CCL) catalyzes the cleavage of citryl-CoA into oxaloacetate and acetyl-CoA (Aoshima et al. 2004). The sequence of citrate synthase (CS), the enzyme of the oxidative TCA cycle catalyzing the synthesis of citrate, shows similarities to the sequence of CCL, making it quite reasonable to believe that CS, the enzyme of the oxidative cycle, evolved from CCL, the enzyme of the reductive cycle (Aoshima et al. 2004, Hügler et al. 2007). Apart from *H. thermophilus*, the two-enzyme version of citrate

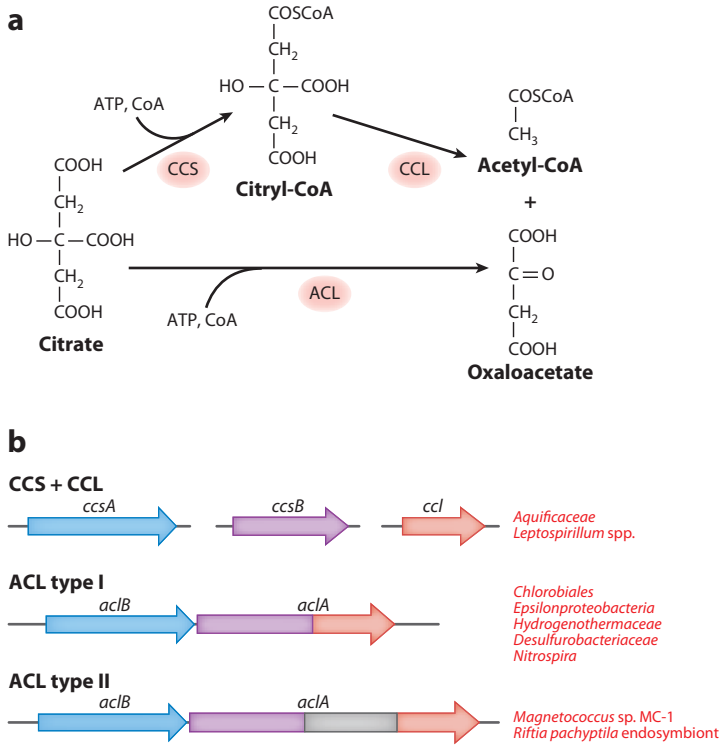


Figure 3

(*a*) Reaction scheme of the citrate cleavage reaction within the reductive tricarboxylic acid cycle. (*b*) Genetic organization and representative groups of organisms harboring the genes (*red*) coding for the different citrate cleaving enzymes: citryl-CoA synthetase (*ccsA* and *ccsB*) and citryl-CoA lyase (*ccl*); bona fide adenosine triphosphate (ATP) citrate lyase (*aclB*, *aclA*); and proposed ATP citrate lyase type II (*aclB*, *aclA*). Abbreviations: CCS, citryl-CoA synthetase; CCL, citryl-CoA lyase; ACL, ATP citrate lyase.

cleavage is also present in other members of the family Aquificaceae (Hügler et al. 2007), and the genes encoding CCS and CCL have also been found in the genome sequences of *Leptospirillum* spp. (Levican et al. 2008). In contrast, in two other families of the Aquificales (Hydrogenothermaceae and Desulfurobacteriaceaea), as well as in Epsilonproteobacteria, Chlorobiales, and "*Ca.* N. defluvii" the cleavage of citrate is catalyzed by a single two-subunit enzyme, ATP citrate lyase (ACL) (Wahlund & Tabita 1997; Hügler et al. 2005, 2007; Takai et al. 2005; Lücker et al. 2010). Because the second subunit of ACL harbors the domain for citryl-CoA cleavage, it is believed that ACL evolved by a gene fusion event of the second subunit of CCS and CCL (Aoshima et al. 2004).

Quite interestingly, within the genome sequence of *Magnetococcus* sp. MC-1, neither CCS and CCL nor ACL are present, although the organism can grow autotrophically by means of the rTCA cycle (Williams et al. 2006). The same holds true for "*Ca.* E. persephone" (Markert et al. 2007). In these cases, a novel type of ACL may be active. The sequence of the postulated type II ACL shows similarities to CCS and CCL as well as to ACL. The second subunit, however, is much longer than the subunit of a bona fide ACL (~900 AA versus ~600 AA), with an insertion between the domains corresponding to CCS and CCL domains (**Figure 3**). Putative type II ACL genes are also found in the genomes of *Methylobacterium nodulans* (Alphaproteobacterium), two *Burkholderia* spp. (Betaproteobacteria), and *Geobacter metallireducens* (Deltaproteobacterium), possibly allowing

these organisms to grow autotrophically by means of the rTCA cycle. It should be emphasized that experimental proof for the citrate cleavage activity of the postulated type II ACL is still missing.

Reductive Acetyl-CoA Pathway

The reductive acetyl-CoA pathway, or Wood-Ljungdahl (WL) pathway, was discovered and elucidated in acetogenic bacteria—anaerobic bacteria, which form acetate from H_2 and CO_2—mainly in the laboratories of Harland G. Wood and Lars G. Ljungdahl (Ljungdahl & Wood 1969, Ljungdahl 1986, Drake et al. 2008, Ljungdahl 2009). It is a relatively simple pathway in which two molecules of CO_2 are combined directly in a noncyclic way to acetyl-CoA. The pathway (**Figure 2*c***) can be divided into two branches: the methyl branch, where CO_2 is consecutively reduced to a cofactor-bound methyl residue, and the carbonyl branch, where another molecule of CO_2 is reduced to an enzyme-bound carbonyl residue. The key enzyme of the pathway is CO dehydrogenase/acetyl-CoA synthase, which catalyzes the reduction of CO_2 to CO as well as the following step, the synthesis of acetyl-CoA from the methyl and the carbonyl residues (Pezacka & Wood 1984, Ragsdale & Wood 1985). The reduction of CO_2 to the methyl group is accomplished by a series of enzymes, most of which are also unique for this pathway (Ljungdahl 1986, Ragsdale & Pierce 2008).

Most acetogens belong to the Gram-positive Clostridiales (see Drake et al. 2008 for a list of species); however, some Spirochaeta also exhibit an acetogenic lifestyle using the WL-pathway (Leadbetter et al. 1999, Pierce et al. 2008). Apart from acetogenic bacteria, which actually have a versatile metabolism and can also grow heterotrophically (Drake et al. 2008), the pathway is used in autotrophic sulfate-reducing bacteria and archaea as well as in methanogenic archaea, and potentially in planctomycetes carrying out the anaerobic oxidation of ammonium (anammox) (Jansen et al. 1984, Zeikus et al. 1985, Schauder et al. 1989, Fuchs 1994, Vorholt et al. 1995, Strous et al. 2006). Thus, the WL-pathway is so far the only carbon fixation pathway present in both bacteria and archaea, in line with the hypothesis that it is the most ancient autotrophic carbon fixation pathway (Fuchs 1989, Martin et al. 2008, Berg et al. 2010a). Yet, distinct variants of the pathway exist in the two domains. Whereas formate is a free intermediate in bacteria, formyl-methanofuran is formed in methanogenic archaea. In addition, the C_1 carriers involved—tetrahydrofolate in bacteria, tetrahydropterins in archaea—are different, and thus so are the enzymes involved in the formation of the cofactor-bound methyl group. Only the key enzyme CO dehydrogenase/acetyl-CoA synthase appears to have the same origin in bacteria and archaea (Berg et al. 2010a).

3-Hydroxypropionate Bicycle

The 3-hydroxypropionate bicycle (also 3-HP/malyl-CoA cycle) operates in the green nonsulfur bacterium *Chloroflexus aurantiacus* and most likely in other members of Chloroflexaceae as well (Strauss & Fuchs 1993, Herter et al. 2002b, Zarzycki et al. 2009). Initially, Helge Holo questioned the operation of the CBB cycle in *C. aurantiacus* and suggested a novel CO_2 fixation pathway with 3-hydroxypropionate as intermediate (Holo 1989). Yet, it took another 20 years of research in the laboratory of Georg Fuchs to completely elucidate this pathway (Zarzycki et al. 2009 and references therein).

As shown in **Figure 2*b***, two cycles are involved in this CO_2 fixation pathway and, consequently, the name 3-HP bicycle has now been proposed (Zarzycki et al. 2009). In the first cycle, two molecules of bicarbonate are fixed and glyoxylate is formed as the first CO_2 fixation product. In the second cycle, glyoxylate and propionyl-CoA are disproportionated to pyruvate and acetyl-CoA (Herter et al. 2002b, Zarzycki et al. 2009). In summary, one molecule of pyruvate is formed from

three molecules of bicarbonate, involving the carboxylating enzymes acetyl-CoA and propionyl-CoA carboxylase.

Only 13 enzymes catalyze the 19 reactions of the pathway due to the involvement of several multifunctional enzymes, including malonyl-CoA reductase, propionyl-CoA synthase, and malyl-CoA/β-methylmalyl-CoA/citramalyl-CoA (MMC) lyase, which can be considered key enzymes for the 3-HP bicycle (Zarzycki et al. 2009). Malonyl-CoA reductase catalyzes the two-step reduction of malonyl-CoA to 3-hydroxypropionate, the characteristic intermediate of the pathway (Hügler et al. 2002). Subsequently, the trifunctional enzyme propionyl-CoA synthase transforms 3-hydroxypropionate to propionyl-CoA (Alber & Fuchs 2002). The third key enzyme, MMC lyase, catalyzes three different reactions: (*a*) the cleavage of malyl-CoA to acetyl-CoA and glyoxylate, (*b*) the condensation of glyoxylate with propionyl-CoA, forming methylmalonyl-CoA, and (*c*) the cleavage of citramalyl-CoA to pyruvate and acetyl-CoA (Herter et al. 2002a, Zarzycki et al. 2009). Other characteristic enzymes of the 3-HP bicycle are two CoA-transferases and two hydratases (**Figure 2*b***) (Zarzycki et al. 2009). Until now, the 3-HP bicycle appears to be restricted to Chloroflexaceae. Apart from this, single genes of the pathway have been detected in various strains of Alpha- and Gammaproteobacteria, yet the complete gene complement is missing (Zarzycki et al. 2009).

3-Hydroxypropionate/4-Hydroxybutyrate Cycle

The 3-HP/4-HB cycle operates in autotrophic thermoacidophilic members of the crenarchaeal order Sulfolobales (Berg et al. 2007, 2010b). Initially, Kandler & Stetter (1981) suggested an undefined reductive carboxylic acid pathway for *Sulfolobus brierleyi* (now *Acidianus brierleyi*) based on $^{14}CO_2$-labeling studies. A few years later, the discovery of acetyl-CoA carboxylase activities in *Sulfolobus* spp. (upregulated in autotrophically grown cells) (Norris et al. 1989) came by surprise, as archaea lack fatty acids in their membranes and thus do not need this enzyme for fatty acid biosynthesis. Hence, this carboxylase could be involved in carbon fixation, as was already known for the 3-HP bicycle of *C. aurantiacus*, and indeed, enzymatic studies suggested the operation of a modified 3-HP cycle in *A. brierleyi* (Ishii et al. 1997); this was also confirmed for other members of Sulfolobales, including the model organisms *Metallosphaera sedula* (Ménendez et al. 1999, Hügler et al. 2003a). However, because malyl-CoA lyase activity was absent (Hügler et al. 2003a), the regeneration of acetyl-CoA remained unsolved until Berg et al. (2007) suggested a novel option involving 4-hydroxybutyrate as intermediate and thus reported the outline of the 3-HP/4-HB cycle (**Figure 2*d***).

The first part of the 3-HP/4-HB cycle, the reaction sequence from acetyl-CoA to succinyl-CoA, is identical to the 3-HP bicycle of *C. aurantiacus*. However, in *M. sedula*, the transformation of malonyl-CoA to propionyl-CoA involves five different enzymes (Berg et al. 2010a and references therein), whereas in *C. aurantiacus*, only two multifunctional enzymes are required (Alber & Fuchs 2002, Hügler et al. 2002). Furthermore, the genes coding for the *M. sedula* enzymes show no sequence similarities to the genes of *C. aurantiacus*, suggesting a separate evolution of the pathway in Sulfolobales and Chloroflexaceae (Berg et al. 2010a). The second part of the 3-HP/4-HB cycle, the regeneration of acetyl-CoA from succinyl-CoA, clearly differs from the 3-HP bicycle. Succinyl-CoA is transformed to acetoacetyl-CoA, which is then cleaved into two molecules of acetyl-CoA (Berg et al. 2007). The most significant enzyme of the 4-HB part of the cycle is 4-hydroxybutyryl-CoA dehydratase, forming crotonyl-CoA from 4-hydroxybutyryl-CoA (Berg et al. 2007). Up to this point, the enzyme was only known to act in a few anaerobic bacteria that ferment 4-aminobutyrate (Buckel & Golding 2006). Several enzymes of the 3-HP/4-HB cycle have been studied in *M. sedula* (Berg et al. 2010a and references therein). Quite interestingly,

some enzymes are promiscuous, e.g., acetyl-CoA/propionyl-CoA carboxylase, which catalyzes both carboxylation reactions (Hügler et al. 2003b), or malonyl-CoA/succinyl-CoA reductase, an enzyme reducing malonyl-CoA and succinyl-CoA to the respective semialdehydes (Alber et al. 2006, Kockelkorn & Fuchs 2009).

The 3-HP/4-HB cycle seems to be used in all autotrophic members of the Sulfolobales, not only in microaerophilic strains but also in the strictly anaerobic *Stygiolobus azoricus* (Berg et al. 2010b). Although all enzyme activities of the complete pathway have been confirmed for only *M. sedula* and *S. azoricus* (Berg et al. 2007, 2010b), key enzymes or genes have been detected in several *Acidianus* spp. and *Sulfolobus* spp. (Ishii et al. 1997, Ménendez et al. 1999, Hügler et al. 2003a, Berg et al. 2010b). In addition, the genome sequences of the mesophilic marine group 1 Crenarchaeota, *Cenarchaeum symbiosum* and "*Ca.* Nitrosopumilus maritimus," suggest the operation of a variant of the 3-HP/4-HP cycle in this ecologically important group discussed below (Hallam et al. 2006, Walker et al. 2010).

Dicarboxylate/4-Hydroxybutyrate Cycle

The outlines of the sixth CO_2 fixation pathway, the dicarboxylate/4-hydroxybutyrate (DC/4-HB) cycle, were described only recently by Huber et al. (2008). The pathway was elucidated in the thermophilic crenarchaeon *Ignicoccus hospitalis* (Desulfurococcales), but it is also present in other Crenarchaeota (Ramos-Vera et al. 2009, Berg et al. 2010b). In 2003, a survey of carbon-fixation enzyme activities in different Crenarchaeaota led to the suggestion of a novel CO_2 fixation pathway in *Ignicoccus* spp. (Hügler et al. 2003a). Later, Jahn et al. (2007) proposed pyruvate synthase and PEP carboxylase as carboxylating enzymes for the novel pathway. Only after the discovery of the 3-HP/4-HB cycle did it become clear that *I. hospitalis* uses the same reaction sequence for the formation of acetyl-CoA from succinyl-CoA, and the pathway was completely solved (Huber et al. 2008).

The DC/4-HB cycle (**Figure 2*e***) partly involves enzymes of the rTCA cycle (the enzymes converting oxaloacetate to succinyl-CoA) as well as enzymes of the 4-HB part of the 3-HP/4-HB cycle (the enzymes converting succinyl-CoA to acetyl-CoA). Three additional enzymes (pyruvate synthase, pyruvate:water dikinase, and PEP carboxylase) are required in order to convert acetyl-CoA to oxaloacetate. Quite interestingly, the DC/4-HB cycle has no enzymes that are unique to this pathway, necessitating the measurement of a combination of enzymes to be able to determine the operation of this cycle.

Because many enzymes of the rTCA cycle are also required for the DC/4-HB cycle, early investigations in the crenarchaeon *T. neutrophilus* (Thermoproteales) suggested the operation of the rTCA cycle in this organism (e.g., Beh et al. 1993). However, reinvestigations confirmed the usage of the DC/4-HB cycle (Ramos-Vera et al. 2009). In addition, the genes of the cycle are present within the genomes of other Thermoproteales (e.g., *Pyrobaculum islandicum*, *P. calidifontis*) (Ramos-Vera et al. 2009). Only recently have enzyme activity measurements also confirmed the operation of the DC/4-HB cycle in *Pyrolobus fumarii* (Desulfurococcales) (Berg et al. 2010b). Thus, presently, it looks as if all autotrophic members of the crenarchaeal orders Desulfurococcales and Thermoproteales use this pathway for carbon fixation.

ENVIRONMENTAL RELEVANCE AND ECOLOGY OF ORGANISMS USING ALTERNATIVE CARBON FIXATION PATHWAYS

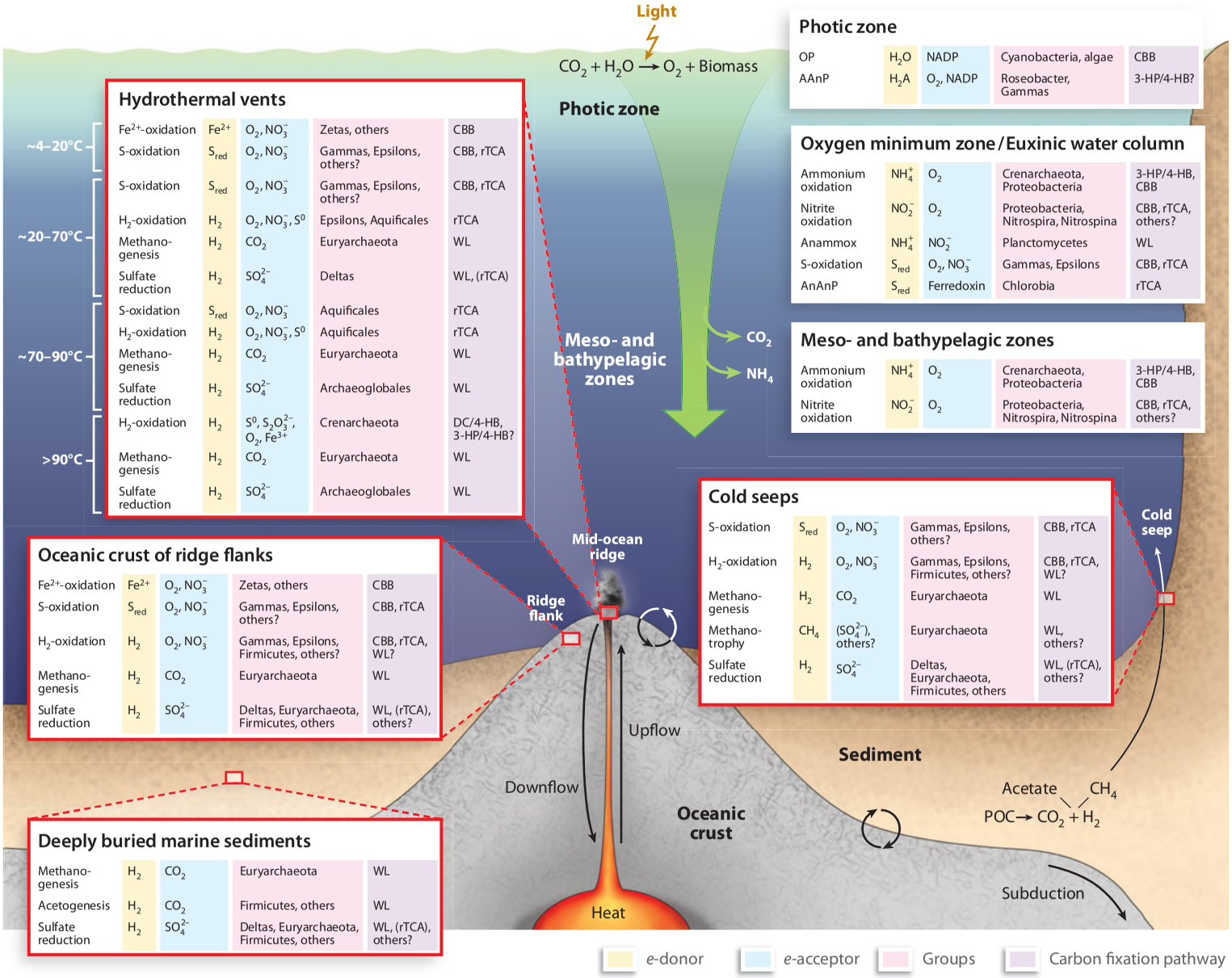
In the following section, we have selected a number of case studies to highlight the environmental relevance and ecology of organisms using alternative carbon fixation pathways in the ocean (**Figure 4**) (see also the sidebar Functional Gene Approaches, below).

FUNCTIONAL GENE APPROACHES

With some exceptions, the phylogenetic distribution of the different carbon fixation pathways makes it impossible to infer the carbon fixation pathway used by a particular organism solely from 16S rRNA gene data. In addition, autotrophic organisms are not necessarily the most abundant community members, making their detection based on 16S rRNA gene approaches problematic. To circumvent these and other issues, functional gene approaches are powerful tools to assess the diversity, abundance, and activity of organisms with specific functions. Since the pioneering work by Paul et al. (1990), numerous studies have been carried out based on the detection of genes coding for subunits of RubisCO to detect organisms utilizing the Calvin-Benson-Bassham cycle for carbon fixation in the environment (see Witte et al. 2010 for a recent example). In contrast, functional gene approaches for alternative carbon fixation have only more recently been developed. Approaches to assess the diversity of microorganisms using the rTCA for carbon fixation pathways were first developed by Campbell et al. (2003) and were subsequently used in a number of studies, primarily at deep-sea hydrothermal vents (e.g., Campbell & Cary 2004, Perner et al. 2007, Voordeckers et al. 2008, Hügler et al. 2010). At present, no functional gene approaches exist to specifically assess organisms utilizing the reductive acetyl-CoA pathway or the 3-HP/4-HB and DC/4-HB cycles. However, approaches based on the detection of an archaeal biotin-dependent acetyl-CoA/propionyl-CoA carboxylase have been used to assess the diversity and distribution of chemoautotrophic crenarchaea that use the 3-HP/4-HB cycle (Auguet et al. 2008, Yakimov et al. 2008) because these carboxylating enzymes have been proposed as indicators for autotrophic metabolism in archaea due to the absence of fatty acids (Ménendez et al. 1999). In addition, a functional gene approach based on the gene coding for 4-hydroxybutyryl-CoA dehydratase to detect organisms using either the 3-HP/4-HB or the DC/4-HB cycle appears feasible.

Case Study 1: Deep-Sea Hydrothermal Vents

Deep-sea hydrothermal vents were first discovered in 1977 on the Galapagos rift (Lonsdale 1977, Corliss et al. 1979) and were the first ecosystems to be identified where chemoautotrophic production is the predominant form of organic carbon production (Jannasch & Wirsen 1979, Jannasch & Mottl 1985). Even today, vents remain the poster child for illustrating the importance of chemoautotrophic processes. Hydrothermal vents are commonly located along mid-ocean ridges, where ocean plates are spreading apart due to an underlying magma chamber, but can also be found in other settings such as back-arc spreading centers, hot-spot volcanoes, sea mounts, and off-axis locations, with distinct differences in the discharged hydrothermal fluids that are manifest in the resulting biological communities (Kelley et al. 2002, Reysenbach & Shock 2002, Schrenk et al. 2010). The hydrothermal fluids that form through seawater-rock interactions within the hydrothermal system are highly enriched in reduced chemical species, e.g., H_2, H_2S, Fe^{2+}, or methane, with the actual composition and concentration depending on the geological setting (Kelley et al. 2002). The mixing of these so-called geofuels (Bach et al. 2006) with cold, oxygenated deep-sea water either above or below the seafloor creates chemical disequilibria that can be harnessed by a diverse array of metabolically versatile chemolithoautotrophic microorganisms, producing organic matter that forms the base of the food chains in these highly productive ecosystems (**Figure 4**). Thus, microbes effectively transfer the energy from the geothermal source to the higher trophic levels (Jannasch & Mottl 1985). Although the validity of this conceptual framework is well established at this point, there are still significant gaps in our understanding of the kinds of microorganisms mediating these reactions in different geothermal systems, the metabolic pathways employed by the microbes, the rates of the catalyzed reactions, the amount of carbon produced, and the larger role of these ecosystems in global biogeochemical cycles.



Photic zone

OP	H_2O	NADP	Cyanobacteria, algae	CBB
AAnP	H_2A	O_2, NADP	Roseobacter, Gammas	3-HP/4-HB?

Oxygen minimum zone / Euxinic water column

Ammonium oxidation	NH_4^+	O_2	Crenarchaeota, Proteobacteria	3-HP/4-HB, CBB
Nitrite oxidation	NO_2^-	O_2	Proteobacteria, Nitrospira, Nitrospina	CBB, rTCA, others?
Anammox	NH_4^+	NO_2^-	Planctomycetes	WL
S-oxidation	S_{red}	O_2, NO_3^-	Gammas, Epsilons	CBB, rTCA
AnAnP	S_{red}	Ferredoxin	Chlorobia	rTCA

Meso- and bathypelagic zones

Ammonium oxidation	NH_4^+	O_2	Crenarchaeota, Proteobacteria	3-HP/4-HB, CBB
Nitrite oxidation	NO_2^-	O_2	Proteobacteria, Nitrospira, Nitrospina	CBB, rTCA, others?

Hydrothermal vents

Fe^{2+}-oxidation	Fe^{2+}	O_2, NO_3^-	Zetas, others	CBB
S-oxidation	S_{red}	O_2, NO_3^-	Gammas, Epsilons, others?	CBB, rTCA
S-oxidation	S_{red}	O_2, NO_3^-	Gammas, Epsilons, others?	CBB, rTCA
H_2-oxidation	H_2	O_2, NO_3^-, S^0	Epsilons, Aquificales	rTCA
Methano-genesis	H_2	CO_2	Euryarchaeota	WL
Sulfate reduction	H_2	SO_4^{2-}	Deltas	WL, (rTCA)
S-oxidation	S_{red}	O_2, NO_3^-	Aquificales	rTCA
H_2-oxidation	H_2	O_2, NO_3^-, S^0	Aquificales	rTCA
Methano-genesis	H_2	CO_2	Euryarchaeota	WL
Sulfate reduction	H_2	SO_4^{2-}	Archaeoglobales	WL
H_2-oxidation	H_2	S^0, $S_2O_3^{2-}$, O_2, Fe^{3+}	Crenarchaeota	DC/4-HB, 3-HP/4-HB?
Methano-genesis	H_2	CO_2	Euryarchaeota	WL
Sulfate reduction	H_2	SO_4^{2-}	Archaeoglobales	WL

Cold seeps

S-oxidation	S_{red}	O_2, NO_3^-	Gammas, Epsilons, others?	CBB, rTCA
H_2-oxidation	H_2	O_2, NO_3^-	Gammas, Epsilons, Firmicutes, others?	CBB, rTCA, WL?
Methano-genesis	H_2	CO_2	Euryarchaeota	WL
Methano-trophy	CH_4	(SO_4^{2-}), others?	Euryarchaeota	WL, others?
Sulfate reduction	H_2	SO_4^{2-}	Deltas, Euryarchaeota, Firmicutes, others	WL, (rTCA), others?

Oceanic crust of ridge flanks

Fe^{2+}-oxidation	Fe^{2+}	O_2, NO_3^-	Zetas, others	CBB
S-oxidation	S_{red}	O_2, NO_3^-	Gammas, Epsilons, others?	CBB, rTCA
H_2-oxidation	H_2	O_2, NO_3^-	Gammas, Epsilons, Firmicutes, others?	CBB, rTCA, WL?
Methano-genesis	H_2	CO_2	Euryarchaeota	WL
Sulfate reduction	H_2	SO_4^{2-}	Deltas, Euryarchaeota, Firmicutes, others	WL, (rTCA), others?

Deeply buried marine sediments

Methano-genesis	H_2	CO_2	Euryarchaeota	WL
Acetogenesis	H_2	CO_2	Firmicutes, others	WL
Sulfate reduction	H_2	SO_4^{2-}	Deltas, Euryarchaeota, Firmicutes, others	WL, (rTCA), others?

Hydrothermal systems have prevailed throughout Earth's history and they may resemble sites where life originated (Baross & Hoffman 1985, Nisbet & Sleep 2001, Martin et al. 2008). Even today there are parts of the hydrothermal system that may be truly independent of photosynthesis, similar to the conditions on early Earth. Processes that fall into this category are hydrogenotrophic methanogenesis, sulfur reduction, and sulfur disproportionation. However, it is important to realize that many chemolithoautotrophic processes occurring at vents (and in the ocean in general) today depend on the presence of oxygen or other oxidized electron acceptors, e.g., nitrate and sulfate, ultimately linking a significant amount, if not most, of the organic matter production at vents (and in other parts of the ocean) to oxygenic photosynthesis. Interestingly, these chemoautotrophs couple the consumption of oxygen to the formation of new biomass rather than to degrading organic matter, with implications for the overall redox state of Earth as well as the balance between O_2 and CO_2 (Schrenk et al. 2010).

Based on thermodynamic and bioenergetic calculations, the estimated carbon production at deep-sea vents is in the range of 5 Tg C y^{-1} (Jannasch 1995, McCollom & Shock 1997). However, it is not known how much organic carbon is being produced in the different habitats, e.g., above versus below the seafloor, and ongoing research aims to determine which pathway contributes how much to the overall carbon production. Presently available data suggest that the CBB cycle and the rTCA cycle are the predominant carbon fixation pathways at deep-sea vents (Campbell et al. 2006, Nakagawa & Takai 2008, Sievert et al. 2008a). The rTCA cycle appears to be the dominant pathway in habitats characterized by temperatures between 20°C and 90°C, whereas the CBB cycle and other carbon fixation pathways, such as the WL-pathway or the DC/4-HB cycle, may be the principal pathways <20°C and >90°C, respectively.

The rTCA cycle operates in Aquificales and Epsilonproteobacteria (Beh et al. 1993; Hügler et al. 2005, 2007; Takai et al. 2005), both of which have been found to be important, if not dominant, community members at a variety of deep-sea hydrothermal vent sites (Reysenbach & Shock 2002, Campbell et al. 2006, Nakagawa & Takai 2008). The main growth temperature for Aquificales, which fall into the families Aquificaceae, Hydrogenothermaceae, and Desulfurobacteriaceae, is between 60°C and 90°C, with some members able to grow at temperatures above 90°C. Whereas Aquificaceae and Hydrogenothermaceae contain mainly microaerophilic chemolithoautotrophs that obtain energy by oxidizing molecular hydrogen or reduced sulfur compounds, all members of the Desulfurobacteriaceae are strict anaerobes, obtaining energy by coupling the oxidation of H_2 to the reduction of either elemental sulfur or nitrate (Nakagawa & Takai 2008). Aquificales seem to be associated mainly with sulfide structures, and they most likely colonize the subsurface portion of vents as well (e.g., Harmsen et al. 1997, Reysenbach et al. 2000, Huber et al. 2003, Voordeckers et al. 2008).

Figure 4

Schematic diagram illustrating where autotrophic processes occur and which particular organisms using alternative carbon fixation pathways play a prominent role in the ocean. Relevant carbon fixation pathways, apart from the Calvin-Benson-Bassham cycle (CBB), are the reductive tricarboxylic acid cycle (rTCA), the Wood-Ljungdahl pathway (WL), the 3-hydroxypropionate/4-hydroxybutyrate cycle (3-HP/4-HB), and the dicarboxylate/4-hydroxybutyrate cycle (DC/4-HB). Recently, it became clear that these pathways provide important contributions to carbon fixation in many oceanic environments, most notably deep-sea hydrothermal vents, cold seeps, the meso- and bathypelagic ocean, oxygen deficiency zones, redoxclines, and euxinic waters. Of particular relevance are the rTCA cycle, which appears to be the main carbon fixation pathway at deep-sea vents, the 3-HP/4-HB cycle most likely operating in nitrosofying crenarchaea in the dark pelagic realm, and the WL-pathway operating in methanogenic archaea and potentially in anammox-catalyzing planctomycetes. Abbreviations: AnAP, anoxygenic aerobic photosynthesis; OP, oxygenic photosynthesis; anammox, anaerobic ammonium oxidation; S-oxidation, sulfur oxidation; Fe^{2+}-oxidation, iron oxidation; S_{red}, reduced sulfur compounds; Gammas, Gammaproteobacteria; Epsilons, Epsilonproteobacteria; Zetas, Zetaproteobacteria; POC, particulate organic carbon.

Epsilonproteobacteria have been identified in a number of studies as important members of the microbial communities at deep-sea hydrothermal vents, including free-living bacterial populations in vent fluids, black-smoker chimney walls, surfaces exposed to hydrothermal fluids, and the shallow subsurface (Campbell et al. 2006 and references therein, Huber et al. 2007). Epsilonproteobacteria exhibit metabolisms similar to that of the Aquificales—i.e., the oxidation of reduced sulfur compounds and hydrogen with both oxygen and nitrate or the oxidation of hydrogen with elemental sulfur coupled to the fixation of inorganic carbon—and thus occupy a similar ecological niche but at lower temperatures, between 20°C and 70°C (Campbell et al. 2006). Different groups of Epsilonproteobacteria may occupy different niches. Members of the *Nautilia/Caminibacter* group grow at temperatures between 40°C and 70°C, and they rely on hydrogen oxidation coupled to the reduction of nitrate or elemental sulfur (S°) as the energy source. In contrast, members of the *Sulfurimonas* group and the *Sulfurovum* group grow at lower temperatures, between 10°C and 40°C, and use reduced sulfur compounds and/or molecular hydrogen as electron donors and oxygen or nitrate as electron acceptors (Campbell et al. 2006). Compared with other epsilonproteobacterial isolates, members of these groups can tolerate relatively high amounts of oxygen (Nakagawa et al. 2005). These metabolic features may be beneficial in ecosystems where intensive mixing of hydrothermal fluid and seawater takes place, like the shallow subsurface, and indeed, Epsilonproteobacteria related to *Sulfurovum* and *Sulfurimonas* spp. are often found to dominate such ecosystems (Campbell et al. 2006, Huber et al. 2007, Hügler et al. 2010). *Arcobacter* and related Epsilonproteobacteria, also frequently found in vent ecosystems (e.g., Moussard et al. 2006, Huber et al. 2007), may require higher sulfide concentrations (Sievert et al. 2007, Wirsen et al. 2002).

Ammonium oxidation, e.g., carried out aerobically by Crenarchaeota likely using the 3-HP/4-HB cycle and anaerobically by planctomycetes using the WL-pathway for carbon fixation, is another process potentially contributing to chemoautotrophic production at vents (Byrne et al. 2009; Wang et al. 2009a,b). With autotrophic organisms catalyzing the reduction of nitrate to N_2 or ammonium (e.g., Epsilonproteobacteria and Aquificales) and methanogens fixing N_2 (Mehta & Baross 2006), the potential for a complete nitrogen cycle carried out solely by autotrophic organisms exists at vents.

Evidence has been obtained for microbial growth at temperatures up to approximately 120°C and for survival up to 127°C (Blöchl et al. 1997, Kashefi & Lovley 2003, Takai et al. 2008), but it is conceivable that microbial growth may occur at temperatures up to 140°C (Holden & Daniel 2004). Because mainly hyperthermophilic archaea grow at temperatures above 90°C, most if not all of the biomass production at these temperatures is carried out by archaea using alternative carbon fixation pathways. Among the latter are autotrophic methanogens (e.g., *Methanopyrus kandleri*, *Methanocaldococcus jannaschii*), which have also been shown to contribute to primary production in the subseafloor (Baross et al. 1982, Jannasch & Mottl 1985, Takai et al. 2004), and sulfate-reducing euryarchaeota of the genus *Archaeoglobus*; both groups use the WL-pathway for carbon fixation. Also, members of the Crenarchaeota (e.g., *Ignicoccus* spp. and *Pyrolobus fumarii*) that use the DC/4-HB cycle for carbon fixation (Huber et al. 2008, Berg et al. 2010b) grow at temperatures above 90°C. Quite interestingly, *Ignicoccus* spp. gain energy by reducing elemental sulfur with hydrogen, a very simple and probably quite ancient type of metabolism, in line with their potentially ancient carbon fixation pathway (Berg et al. 2010a). The use of the 3-HP/4-HB cycle by hyperthermophilic Crenarchaeota has so far been reported in only acidophilic members of the Sulfolobales. Members of this group have not yet been detected at deep-sea vents, not even in the low-pH (1.6) vents of the TOTO caldera in the Mariana Volcanic Arc, although it has been speculated that the so far uncultured deep-sea hydrothermal vent (DHVEG) group could play this role (Nakagawa et al. 2006). It is worth adding that the WL-pathway could also contribute

considerably to chemoautotrophic production at lower temperatures by operating in mesophilic methanogens, sulfate reducers, and anammox catalyzing planctomycetes.

Symbiosis. Symbiotic associations between chemoautotrophic bacteria and animals are prevalent at deep-sea hydrothermal vents, and a large proportion of the biomass at vents is produced by these symbionts nourishing their hosts (Cavanaugh 1994, Dubilier et al. 2008). Many endosymbionts at vents belong to the Gammaproteobacteria and use the CBB cycle for carbon fixation (Nelson & Fisher 1995, Dubilier et al. 2008). Quite unexpectedly, recent proteomic and enzymatic studies suggest that the rTCA cycle operates in addition to the CBB cycle in the gammaproteobacterial endosymbiont of the giant tubeworm *Riftia pachyptila* (Markert et al. 2007). Because tubeworms dominate the vent fauna at vent sites in the Pacific Ocean and different species of vent tubeworms (e.g., *Riftia*, *Tevnia*, and *Ridgeae*) most likely harbor the same symbiont (e.g., Nelson & Fisher 1995), this suggests that the rTCA cycle could also contribute quite significantly to primary production in such symbiotic communities. In addition, the epsilonproteobacterial endosymbiont of gastropods occurring at deep-sea vents in the Indian Ocean has been shown to use the rTCA cycle (e.g., Suzuki et al. 2005). Furthermore, Epsilonproteobacteria are important members of the epibiotic community on vent animals, such as shrimp (e.g., *Rimicaris exoculata*), crabs (e.g., *Kiwa hirsuta*), barnacles (e.g., *Vulcanolepas osheai*), and polychaetes (e.g., *Alvinella pompejana*) (reviewed in Goffredi 2010). Similar to the free-living Epsilonproteobacteria, the animals harboring Epsilonproteobacteria as symbionts are usually found in warmer, more reducing high-flux areas (e.g., Suzuki et al. 2005, Schmidt et al. 2008, Podowski et al. 2009). In summary, these observations demonstrate that we are just at the beginning of elucidating the complex mechanisms driving symbiotic interactions, and even basic features such as the carbon metabolism of the symbionts still reveal surprises.

Stable carbon isotope studies. Different carbon fixation pathways result in distinct isotopic signatures of the produced biomass due to the isotopic discrimination between light (^{12}C) and heavy (^{13}C) carbon by the carboxylating enzymes (Fuchs 1989, House et al. 2003, Schouten et al. 2004, Berg et al. 2010a). Thus, inferences about the carbon fixation pathway predominantly utilized by the microbial community can also be made based on the stable carbon isotopic composition of the organic matter (e.g., Jahnke et al. 2001, Zhang et al. 2004), although one has to keep in mind that a number of other environmental factors contribute to the overall isotopic composition. The observation of distinct isotopic signals of microbial biomass and animals either living in symbiosis or feeding on primary producers goes back to the early days of the discovery of vents and has been used as proof for local, chemoautotrophic production—the "You are what you eat" concept (Rau & Hedges 1979, Van Dover & Fry 1994). Basically two distinct isotopic signals exist at vents: on one hand, heavy biomass with a composition of approximately $^{-}10$–$^{-}15‰$ and, on the other hand, light biomass with an isotopic signal of $^{-}20$–$^{-}30‰$. Whereas the lighter values can easily be explained by the operation of the CBB cycle, the explanation of the heavy values remains difficult. For a long time, only the CBB cycle was considered to be the major carbon fixation pathway: Explanations ranging from carbon limitation to the use of different forms of RubisCO with distinct isotopic fractionation factors have been invoked (Fisher et al. 1990, Van Dover 2002, Robinson et al. 2003, Scott 2003). Although these factors do play a role, it is now clear that the use of alternative carbon fixation pathways, in particular the rTCA cycle, has to be taken into consideration when explaining the heavy isotope values. Along these lines, it would be expected that the isotopic values go from heavy to light along a temperature gradient from zones characterized by a higher hydrothermal fluid flux and thus higher temperatures to the surrounding cooler areas, corresponding to the physiology of the microorganisms utilizing either the rTCA or the CBB cycle for carbon fixation.

The use of the rTCA cycle also provides a more parsimonious explanation for the heavy stable carbon isotopic composition of the tubeworm *Riftia pachyptila* (Markert et al. 2007) and possibly the vent shrimp *Rimicaris exoculata*, similar to what has been found for gastropods (e.g., Suzuki et al. 2005). There is also evidence for selective feeding by hydrothermal vent fauna on different chemoautotrophs utilizing different carbon fixation pathways, e.g., the rTCA cycle and the CBB cycle by Epsilon- and Gammaproteobacteria, respectively (Levin et al. 2009).

Case Study 2: Autotrophic Processes in the Pelagic Realm

Epipelagic zone. Most primary production in the open ocean takes place in the epipelagic zone (upper ~200 m) where sunlight provides enough energy for photosynthesis (**Figure 4**). Thus, the biology of the photic zone differs considerably from the waters of the deep, dark ocean, the meso- and bathypelagic zones. Cyanobacteria constitute an important fraction of the primary producers, performing oxygenic photosynthesis and using the CBB cycle for carbon fixation, although some cyanobacteria actually may not perform either process (Zehr et al. 2008). In addition to chlorophyll-containing organisms (phototrophic eukaryotes and cyanobacteria), bacteria containing bacteriochlorophyll-*a*, the aerobic anoxygenic photosynthetic bacteria (AAnPB), are ubiquitous in the photic zone (Kolber et al. 2001). Initial studies indicated that members of the genus *Erythrobacter* were the main types of AAnPB in the ocean, but subsequent studies found that AAnPB are not restricted to this genus and that other groups are more important, notably members of the *Roseobacter* group (Alphaproteobacteria) and Gammaproteobacteria belonging to the NOR5/OM60 group (Beja et al. 2002, Cho & Giovannoni 2004, Yan et al. 2009). Traditionally, AAnPB are seen as heterotrophs that supplement their energy needs by using light, making them particular efficient in oligotrophic settings (Kolber et al. 2001, Moran & Miller 2007). However, recent genomic data suggest that at least some strains actually may have the capability to grow autotrophically or exhibit a mixotrophic carbon metabolism (Moran & Miller 2007, Swingley et al. 2007, Tang et al. 2009). Interestingly, genes of the 3-HP bicycle are found within AAnPB (Zarzycki et al. 2009). For example, some gammaproteobacterial strains of the NOR5/OM60 clade (*Congregibacter litoralis*, strains NOR5-3, NOR51-B) and *Erythrobacter* sp. NAP1 possess the genes for malonyl-CoA reductase and/or propionyl-CoA synthase and, thus, most likely only a rudimentary version of the 3-HP bicycle. In contrast, the genetic outfit for a complete cycle is present within the genome of strain HTCC2080, also a member of the NOR5/OM60 group (Cho & Giovannoni 2004). Interestingly, the regeneration of acetyl-CoA seems to proceed via 4-hydroxybutyrate (as in Crenarchaeota) and not via malyl-CoA (as in *Chloroflexus*). Thus, strain HTCC2080 may be able to grow autotrophically via a hybrid 3-HP/4-HB cycle (Zarzycki et al. 2009). Notably, even a rudimentary version of the 3-HP cycle may be beneficial in the epipelagic zone as it allows the mixotrophic assimilation of simple organic compounds such as acetyl-CoA, propionyl-CoA, or 3-hydroxypropionate.

Meso- and bathypelagic zones. The meso- and bathypelagic realms of the ocean represent the largest continuous habitats on Earth, yet we know very little about the kind of microbes living in these zones and the global impact of their activities (Aristegui et al. 2009). Traditionally, the role of microbes in these zones has been seen as that of degraders of organic matter, with a concomitant release of CO_2 taking place. However, this perception has recently been called into question by the finding that planktonic Crenarchaeota belonging to the marine group 1 (MG1), which dominate the prokaryotic cell numbers in this environment (Karner et al. 2001), could actually be autotrophs (Pearson et al. 2001, Wuchter et al. 2003) (**Figure 4**). These findings were substantiated with the isolation of the first pure culture belonging to MG1, "*Ca.* N. maritimus" (Könneke et al. 2005).

"*Ca.* N. maritimus" can grow as a chemolithoautotroph, using the oxidation of ammonium with oxygen as its source for energy and reducing equivalents. Subsequently, Crenarchaeota were identified as the predominant ammonium oxidizers in the ocean (e.g., Wuchter et al. 2006), and their ability to incorporate bicarbonate into their biomass has also been confirmed in the natural environment (Hansman et al. 2009, Herndl et al. 2005, Ingalls et al. 2006). Further, some Crenarchaeota may also be heterotrophs and/or mixotrophs (Ouverney & Fuhrman 2000, Ingalls et al. 2006, Agogue et al. 2008). Overall, Crenarchaeota seem to be major drivers of biogeochemical cycles within the oceans' interior, with important ramifications for the nitrogen and carbon cycles, necessitating a revision of the current paradigm of the role of microbes in these zones (Herndl et al. 2008, Aristegui et al. 2009). An interesting aspect of autotrophic carbon fixation by archaea is that this process may transform relatively labile carbon formed by degradation of organic matter in the water column into a more refractory form. Characteristic biomolecules synthesized by MG1 archaea as membrane constituents accumulate in the sediments and can be used to diagnose the presence of these organisms in the geological record as far back as the Cretaceous (Kuypers et al. 2001). Thus, rather than being cycled back to the surface in the form of CO_2 on a timescale of thousands of years as part of the general ocean circulation system, this carbon fraction may be disengaged from the carbon cycle on much longer timescales. Ammonium oxidation, including by Crenarchaeota, has also been identified as an important process in hydrothermal plumes, further contributing to carbon production in the deep ocean (Lam et al. 2004, Dick & Tebo 2010).

As mentioned above, the likely carbon fixation pathway used by the autotrophic ammonium-oxidizing Crenarchaeota is the 3-HP/4-HB cycle, and the frequent detection of key genes of this pathway in metagenomic databases further confirms its occurrence in so far uncultivated representatives (Berg et al. 2007). It has been estimated that ammonium-oxidizing archaea may fix approximately 400 Tg C y^{-1}, making this cycle significant in the pelagic realm of the ocean (Herndl et al. 2005, Wuchter et al. 2006).

At present, it is still unclear which organisms perform the oxidation of nitrite to nitrate in the ocean. Likely candidates are Deltaproteobacteria of the genus *Nitrospina* that co-occur with MG1 archaea in the Pacific Ocean (Mincer et al. 2007). *Nitrospina* spp. probably use an alternative carbon fixation pathway, e.g., the WL-pathway or the rTCA cycle, as both pathways have been identified in Deltaproteobacteria in contrast to the CBB cycle (Fuchs 1989). The rTCA cycle also appears to be used by *Nitrospira* spp., another group of nitrite oxidizers that has been reported from oceanic environments (Watson et al. 1986, Lücker et al. 2010). Clearly, autotrophy in meso- and bathypelagic waters, as well as mixotrophy in the photic zone, is presently poorly understood and may be inadequately represented in carbon models (Karl 2002, DeLong & Karl 2005, Moran & Miller 2007).

Case Study 3: Oxygen Minimum Zones and Oxic-Anoxic Interfaces

We focus in this section on aspects related to carbon fixation in oxygen minimum zones (OMZs) (see Lam & Kuypers 2011 in this volume for a more comprehensive treatment of the microbial processes occurring in OMZs). In recent years, OMZs have received increased attention as they are expected to increase in response to climate change, with potential deleterious effects to oceanic ecosystems (Keeling et al. 2010). In general, one can distinguish between OMZs in an open ocean setting, where oxygen-deficient waters are sandwiched between oxygen-containing water layers, and in euxinic waters that are characterized by a transition from oxic to anoxic and sulfidic waters, creating oxic-anoxic interfaces or redoxclines. One of the main differences between these settings is the presence of sulfide and other reduced sulfur compounds in euxinic waters and their general absence in OMZs, with its implications for the microbial communities and the processes occurring therein (Walsh et al. 2009, Keeling et al. 2010) (**Figure 4**).

Traditionally, heterotrophic processes were thought to dominate in OMZs, i.e., aerobic respiration and denitrification coupled to the oxidation of organic matter. However, this view dramatically changed with the discovery that the anaerobic oxidation of ammonium (anammox) by a specific group of planctomycetes may be responsible for the majority of nitrogen loss in OMZs (Dalsgaard et al. 2003; Kuypers et al. 2003, 2005). Because anammox is an autotrophic process, a significant portion of inorganic carbon may actually be fixed rather than released. The anammox process is inhibited by oxygen, yet organisms quickly resume their activity once anaerobic conditions are restored (Strous et al. 1999). Genomic and enzymatic studies of anammox bacteria provided initial evidence for usage of the WL-pathway for carbon fixation (Schouten et al. 2004, Strous et al. 2006), making this pathway potentially of wider significance in the pelagic realm, with a potential contribution of up to 3.5 Tg C y^{-1} (Raven 2009). Interestingly, CO dehydrogenase, the key enzyme of the WL-pathway, is very oxygen sensitive and, so far, the WL-pathway is only known to operate in anaerobic organisms growing under highly reducing conditions (Berg et al. 2010a), unlike conditions prevailing in OMZs. Furthermore, the WL-pathway requires a specific set of cofactors and metals (Berg et al. 2010a), which may either not be present or not be readily available in the more oxidizing conditions of OMZs. This could suggest that the planctomycetes carrying out anammox use a modified pathway and/or enzymes or possibly exhibit internal protective mechanisms. In fact, it has been observed that anammox bacteria closely associate with aerobic, ammonium-oxidizing Crenarchaeota or bacteria. Although these organisms compete for the ammonium, their metabolic activity could lower the oxygen concentration in the immediate vicinity of the anammox cells (Lam et al. 2007). In this context, it is interesting that anammox has so far been identified mainly in areas where conditions in the water column may be more reducing, due to the at least periodic presence of reduced sulfur compounds; such areas include the Black Sea, off the Namibian coast, the Gulfo Dulce off Costa Rica, and the Peruvian upwelling region (e.g., Dalsgaard et al. 2003; Kuypers et al. 2003, 2005; Hamersley et al. 2007). Recently, it has been shown that the relative importance of anammox to denitrification, and thus the balance between autotrophic and heterotrophic processes, differs substantially in different regions, which has been attributed to environmental factors such as spatial and temporal variations in the organic matter quality and quantity (Ward et al. 2009).

At the oxic-anoxic interfaces or redoxclines of permanently euxinic water columns (e.g., Black Sea, Cariaco Basin) and temporarily euxinic water columns (e.g., Baltic Sea, fjords), as well as coastal upwelling regions with periodic input of sulfidic waters (e.g., off the Namibian coast), sulfur-oxidizing microorganisms, including anaerobic phototrophs (particularly Chlorobiales using the rTCA cycle), can play important roles (Jorgensen et al. 1991, Vetriani et al. 2003, Lavik et al. 2009, Marschall et al. 2010, Zaikova et al. 2010). In a number of cases, nitrate is used as alternate electron acceptor, further supporting nitrogen losses due to autotrophic processes. Epsilonproteobacteria have been identified as important community members in the chemocline of the Black Sea, Cariaco Basin, and Baltic Sea, where they fix inorganic carbon, presumably via the rTCA cycle, by coupling the oxidation of reduced sulfur compounds to either O_2 or nitrate consumption (e.g., Vetriani et al. 2003, Lin et al. 2006, Jost et al. 2008, Glaubitz et al. 2009). Even in an open ocean setting like that off the coast of Namibia, episodic sulfide intrusions can stimulate blooms of sulfur-oxidizing Epsilon- and Gammaproteobacteria (Lavik et al. 2009). In particular, a group of Gammaproteobacteria closely related to uncultivated endosymbiotic, sulfur-oxidizing bacteria has been identified as an important member of microbial communities in oxygen-deficient waters worldwide (e.g., Vetriani et al. 2003, Lavik et al. 2009, Zaikova et al. 2010), and the group's chemolithoautotrophic potential was recently confirmed (Walsh et al. 2009). Clearly, autotrophic processes play important roles in many oxygen-deficient zones of the world's oceans, and a significant amount of this carbon production appears to be carried out by organisms using alternative carbon fixation pathways.

Other Oceanic Ecosystems

Obviously, there are other oceanic environments where chemolithoautotrophic processes are important, but due to space constraints, they cannot be covered in greater detail here. However, we would like to at least briefly mention them. Foremost in this context are cold seeps and mud volcanoes, where highly reduced fluids of nonhydrothermal origin, often containing methane and other hydrocarbons, are emanating from the seafloor (Jørgensen & Boetius 2007) (**Figure 4**). In these habitats, anaerobic oxidation of methane and also other hydrocarbons coupled to the reduction of sulfate produces H_2S that fuels chemoautotrophic production carried out predominantly by Epsilon- and Gammaproteobacteria, either free-living or in symbiosis (Jørgensen & Boetius 2007, Dubilier et al. 2008). At methane seeps, anaerobic methane oxidizers fix an appreciable amount of inorganic carbon, most likely via the WL-pathway (Jørgensen & Boetius 2007, Wegener et al. 2008). On the other hand, methane-producing archaea are likely to contribute significantly to autotrophic carbon fixation in deep marine sediments (Fry et al. 2008 and references therein), as do autotrophic acetogens (e.g., Heuer et al. 2009) (**Figure 4**). However, similar to the meso- and bathypelagic realms, both groups are reworking organic matter that was initially produced by oxygenic phototrophs by consuming the products of fermentative organic matter degradation, hydrogen and CO_2 (Fry et al. 2008). In contrast, radiolysis of water can potentially support a subseafloor biosphere independent of photosynthesis (e.g., D'Hondt et al. 2009). The basement rocks of the ridge flanks are another important, but so far largely unconstrained, source of chemoautotrophic carbon production (Bach & Edwards 2003, Cowen et al. 2003, Ehrhardt et al. 2007, Mason et al. 2009, Schrenk et al. 2010). In particular, iron oxidation, hydrogen oxidation, and/or sulfide oxidation have been identified as potential energy sources (**Figure 4**), with an estimate of ~1 Tg C y^{-1} that can potentially be produced within the oceanic crust (Bach & Edwards 2003). There is evidence that autotrophic iron-oxidizing Zetaproteobacteria contribute to carbon production in iron-rich hydrothermal systems and basement rocks, potentially using the CBB cycle (Emerson et al. 2007, Kato et al. 2009). Overall, the deep subseafloor biosphere and its impact on global biogeochemical cycles are still underexplored, and many exciting discoveries, including novel microbial metabolisms, are waiting to be made.

SUMMARY POINTS

1. Presently, six different CO_2 fixation pathways are known to operate in microorganisms. Apart from the reductive pentose phosphate, or Calvin-Benson-Bassham (CBB), cycle, these are the reductive tricarboxylic acid cycle (rTCA); the reductive acetyl-CoA, or Wood-Ljungdahl (WL), pathway; the 3-hydroxypropionate (3-HP) bicycle; the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle; and the dicarboxylate/4-hydroxybutyrate (DC/4-HB) cycle. The latter two have been described only quite recently.
2. The rTCA cycle and the WL-pathway have been found to operate in quite diverse groups of microorganisms. The rTCA cycle seems restricted to bacteria and operates in Chlorobiales, Aquificales, Epsilonproteobacteria, Nitrospirae, and single strains of other proteobacterial groups. The WL-pathway may be the most ancient pathway operating in bacteria (Firmicutes, Planctomycetes, Deltaproteobacteria, Spirochaeata) as well as archaea (Euryarchaeota). The distribution of the other pathways seems more restricted, operating in Chloroflexaceae (3-HP bicycle) and Crenarchaeota (3-HP/4-HB cycle, DC/4-HB cycle).

3. Due to a combination of thermodynamics and biochemistry, aerobic chemolithoautotrophs predominantly use the oxygen-tolerant, but energy-demanding, CBB cycle, whereas anaerobic chemolithoautotrophs that are usually energy-limited utilize more energy-efficient, yet oxygen-sensitive, carbon fixation pathways. At hydrothermal vents, there is a clear partition of carbon fixation pathways with respect to temperature that is also a reflection of the presence of oxygen, as high-temperature habitats are generally more oxygen depleted than low-temperature ones.
4. During the last decade, our knowledge about the phylogenetic and environmental distribution of carbon fixation pathways other than the CBB cycle, the so-called alternative carbon fixation pathways, has increased significantly, and it has become clear that they provide a relevant contribution to carbon fixation in a number of oceanic environments, most notably at deep-sea hydrothermal vents, the meso- and bathypelagic ocean, and in oxygen-deficiency zones and redoxclines. Of particular relevance are the rTCA cycle, which appears to be the main carbon fixation pathway at deep-sea vents, the 3-HP/4-HB cycle most likely operating in nitrosofying crenarchaea in the dark pelagic realm, and the WL-pathway operating in methanogenic archaea and potentially in anammox catalyzing planctomycetes.

FUTURE ISSUES

1. Are there autotrophic aerobic anoxygenic photosynthetic bacteria? To what extent do they contribute to carbon fixation in the epipelagic zone?
2. There are a number of chemoautotrophs for which the carbon fixation pathway has not yet been determined, including *Ammonifex degensii*, *Deferribacter* spp., Thermodesulfobacteriaceae, and *Pyrodictium* spp. Interestingly, in addition to the genes of the WL-pathway, the genome of *Ammonifex* contains a RubisCO form III, known so far only from Archaea, as well as a phosphoribulokinase, possibly indicating a functional CBB cycle, with obvious implications for the evolution of the CBB cycle.
3. How does "*Ca.* Endoriftia persephone" regulate the use of the CBB and rTCA cycles? Under what conditions are the pathways used? How common is the presence of multiple carbon fixation pathways in microorganisms?
4. Do anammox catalyzing planctomycetes occurring in OMZs have special adaptations to protect the enzymes of the WL-pathway from exposure to oxygen, and do they have different requirements for cofactors and trace metals?
5. The operation of the 3-HP/4-HB cycle in planktonic crenarchaea needs to be verified using biochemical and enzymatic studies. Mesophilic Crenarchaeota appear to have a modified pathway with novel enzymes different from the enzymes characterized in hyperthermophilic Crenarchaeota.
6. The contributions by chemoautotrophs to carbon production and the utilized pathways in various environments need to be determined. Promising tools in this regard are the application of stable isotope probing combined with functional gene screens and cell identification, as well as metagenomic, transcriptomic, and proteomic approaches. Also, measurements of carbon fixation rates under in situ conditions will be key.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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