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ANNUAL REVIEWS

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Life in the Dark: Phylogenetic and Physiological Diversity of Chemosynthetic Symbioses

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Keywords

chemosymbiosis, host-microbe associations, nutritional interactions, adaptations to symbiosis, metabolism, thiotroph, methanotroph

Abstract

Possibly the last discovery of a previously unknown major ecosystem on Earth was made just over half a century ago, when researchers found teaming communities of animals flourishing two and a half kilometers below the ocean surface at hydrothermal vents. We now know that these highly productive ecosystems are based on nutritional symbioses between chemosynthetic bacteria and eukaryotes and that these chemosymbioses are ubiquitous in both deep-sea and shallow-water environments. The symbionts are primary producers that gain energy from the oxidation of reduced compounds, such as sulfide and methane, to fix carbon dioxide or methane into biomass to feed their hosts. This review outlines how the symbiotic partners have adapted to living together. We first focus on the phylogenetic and metabolic diversity of these symbioses and then highlight selected research directions that could advance our understanding of the processes that shaped the evolutionary and ecological success of these associations.

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Contents

1. INTRODUCTION

Primary productivity supports all life on our planet. For several centuries, biologists assumed that only one form of primary production existed, the production of organic compounds through light-powered photosynthesis. It was therefore a paradigm shift, although not recognized as such at the time, when in the 1880s the Ukrainian microbiologist Sergei Winogradsky discovered a second form of primary production powered by the oxidation of reduced inorganic substrates. Through a series of elegant experiments, Winogradsky (130) first showed that sulfur-oxidizing bacteria use a reduced inorganic compound, hydrogen sulfide, to fuel their growth. Later, Winogradsky demonstrated that ammonium- and nitrite-oxidizing bacteria coupled the energy generated from the oxidation of inorganic compounds to fix carbon dioxide into biomass (131). This process of chemolithoautotrophy, generally known as chemosynthesis, is the only other form of primary production on Earth. Despite Winogradsky's revelation, it took another 90 years and the discovery of deep-sea hydrothermal vents in 1977 for researchers to realize that chemosynthesis not only supports microbial life but also forms the basis for entire animal ecosystems (reviewed in 17, 125).

It is now well established that chemosynthetic bacteria and archaea form intimate symbiotic relationships with both protist and invertebrate hosts (31). In these chemosymbioses, the host delivers a stable supply of the reductants and oxidants that the symbionts need to acquire energy and carbon for building biomass. In return, the symbionts use these resources to support their own growth and provide their hosts with nutrition. Given that the degree to which chemosymbiotic hosts depend on their symbionts for nutrition spans a wide range, from obligate dependence to partial reliance, delineation of chemosymbiosis is not always unequivocal (see the sidebar titled Putative Chemosymbionts). Further complicating efforts to clearly define chemosymbioses, in many cases the metabolic pathways involved in nutritional interactions are poorly understood. In this review, we focus on the phylogenetic and metabolic diversity of chemosynthetic associations in which the symbionts are assumed to contribute to the nutrition of their hosts.

Many chemosynthetic symbionts are chemolithoautotrophs that couple the oxidation of reduced inorganic substrates to the fixation of carbon dioxide. Other chemosynthetic symbionts use organic one-carbon (C_1) compounds, such as methane, as an energy and carbon source and are chemoorganoheterotrophs. In this review we use chemosymbionts as an umbrella term for

PUTATIVE CHEMOSYMBIONTS

Based on omics data, a number of symbionts associated with eukaryotic hosts may be chemosynthetic. These include the sulfate-reducing deltaproteobacteria of the gutless oligochaete *Olavius algarvensis* (65), the shrimp *Rimicaris* (62), and ciliates (6), which may use hydrogen and carbon monoxide as energy sources. Furthermore, omics data provide good support for the chemosynthetic nature of some bacteria that are associated with eukaryotic hosts but whose role in host nutrition is not clear, such as the iron-oxidizing zetaproteobacteria associated with *Rimicaris* (61), the *Campylobacterota* epibionts of *Bathymodiolus* mussels (3) and the vent barnacle *Vulcanolepas* (121), and *Nitrospirota* bacteria and ammonium-oxidizing betaproteobacteria associated with deep-sea sponges (75, 82). Physiological and experimental data are needed to support the predicted ability of these symbionts to generate energy from inorganic substrates and transfer nutrients to their host.

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bacteria and archaea that use energy acquired from the oxidation of reduced inorganic substrates or C_1 compounds to build biomass. This definition includes mixotrophic symbionts that acquire additional or even all of their carbon through the uptake of more complex organic compounds (e.g., compounds with more than one carbon) (see **Table 1** for an overview of these key terms).

2. PHYLOGENETIC DIVERSITY OF CHEMOSYNTHETIC SYMBIONTS

The vast majority of chemosymbionts are members of *Gammaproteobacteria*, but other bacterial clades, as well as archaea, have also established chemosymbioses with eukaryotes. These include members of *Alphaproteobacteria*, such as those that established a symbiosis with the flatworm *Paracatenula* over 500 million years ago (49); members of *Campylobacterota* (formerly *Epsilonproteobacteria*) that associate with the hydrothermal vent shrimp *Rimicaris* (95), snails (8), polychaetes (51), and mussels (3); and methanogenic archaea that form partnerships with ciliates (6, 126) (**Figure 1**; **Supplemental Figure 1**). The diversity of hosts associated with chemosynthetic bacteria is remarkable. They include at least 3 protist groups and 26 animal families from 7 phyla—Porifera, Cnidaria, Platyhelminthes, Annelida, Mollusca, Nematoda, and Arthropoda (31) (**Table 2**). Nearly all chemosymbioses occur in marine habitats, but there are notable exceptions in freshwater environments with high sulfide concentrations; an example is the symbiosis between freshwater amphipods and filamentous, chemosynthetic ectosymbionts that was discovered in the Frasassi cave system in Italy (26, 41). As researchers continue to sample new and understudied regions of the world's oceans, they regularly discover previously unknown symbionts and hosts, indicating a wealth of taxonomic diversity in chemosymbiotic unions waiting to be revealed (e.g., 48, 127).

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The number of chemosymbiont species within a host is generally consistent among individuals of a host species but varies across host groups. Some hosts, like the tube worm *Riftia*, are dominated by only a single bacterial species or phylotype as defined by 16S rRNA gene sequencing. Others, like the shrimp *Rimicaris*, the gutless oligochaete *Olavius*, and the tube worm *Lamellibrachia*, house multiple phylotypes from several phyla (34, 134, 137). Advances in metagenomic sequencing and bioinformatic tools are providing the capacity to move beyond 16S rRNA gene sequencing and to resolve diversity within a single microbial species, and recent studies have revealed that in some animal groups, like *Bathymodiolus* mussels, as many as 16 strains of a single species of sulfur-oxidizing symbionts co-occur within a single host individual (2, 104). It is likely that by harboring multiple symbiont species or strains, these hosts benefit from their metabolic versatility to use the broad range of energy, carbon, and other resources available in the environment (2, 104).

One intriguing question in research on chemosymbiotic diversity is why the vast majority of symbionts belong to only three microbial groups, *Gammaproteobacteria*, *Alphaproteobacteria*, and

Table 1 Key terms for chemosynthetic symbionts

Key term	Definition	Notes
Chemosynthetic symbiont	Umbrella term for all symbionts that obtain energy from the oxidation of reduced inorganic compounds or C_1 compounds. Electron donors: reduced inorganic compounds (e.g., hydrogen sulfide, hydrogen, carbon monoxide) or organic C_1 compounds (e.g., methane). Carbon sources: inorganic (carbon dioxide) or organic (e.g., methane, short-chain fatty acids) compounds.	Some hosts, like sponges, that house chemosynthetic bacteria (among a wealth of microbes) are not traditionally defined as chemosymbiotic because they do not appear to be nutritionally dependent on their chemosynthetic bacteria (see the sidebar titled Putative Chemosymbionts).
Chemolithoautotroph	Chemosynthetic bacteria that obtain energy from the oxidation of inorganic compounds and use inorganic carbon to generate biomass. Electron donors: reduced inorganic sulfur compounds, hydrogen, carbon monoxide. Carbon source: inorganic (carbon dioxide).	Sulfur-oxidizing symbionts of vesicomyid clams (e.g., "*Candidatus* Ruthia magnifica," which is a symbiont of the deep-sea clam *Calyptogena magnifica*) are obligate chemolithoautotrophs. Very few chemosynthetic symbionts are obligate chemolithoautotrophs; most are mixotrophs.
Chemoorganoheterotroph	Chemosynthetic bacteria that obtain energy from the oxidation of organic compounds and use organic carbon to assimilate biomass. Electron donors: C_1 organic compounds (e.g., methane). Carbon sources: organic compounds (e.g., methane).	Methane-oxidizing symbionts of deep-sea snails and bathymodiolin mussels use methane as both an energy and carbon source. Some free-living methane-oxidizing bacteria also fix carbon dioxide, but this has not been shown in chemosynthetic symbionts. Chemoorganoheterotrophs are not considered chemosynthetic if they use organic compounds with more than one carbon as an energy source.
Chemolithoheterotroph	Chemosynthetic bacteria that obtain energy from the oxidation of inorganic compounds and use organic carbon to generate biomass. Electron donors: reduced inorganic compounds (e.g., H_2S). Carbon sources: organic compounds (e.g., short-chain fatty acids).	Sulfur-oxidizing symbionts, "*Candidatus* Kentron," associated with single-cell ciliates from the genus *Kentrophoros*, lack canonical pathways for autotrophic carbon fixation.
Mixotroph	Chemosynthetic bacteria that use both inorganic and organic carbon sources to build biomass.	Sulfur-oxidizing symbionts (e.g., "*Candidatus* Thiosymbion") associated with sediment-dwelling meiofauna (e.g., *Olavius*, *Inanidrilus*, *Astomonema*, Stilbonematidae) fix CO_2 through chemolithoautotrophy and assimilate organic compounds, like acetate and propionate, through chemolithoheterotrophy to build biomass. Most chemosynthetic symbionts are mixotrophs.

Campylobacterota. There are at least seven additional bacterial phyla, *Planctomycetota*, *Aquificota*, *Bacteroidota*, *Chloroflexota*, *Firmicutes*, *Nitrospirota*, and *Verrucomicrobiota*, that also use reduced inorganic substrates and C_1 compounds to power biosynthesis (56). One explanation is that *Gammaproteobacteria*, *Alphaproteobacteria*, and *Campylobacterota* dominate the free-living community of chemosynthetic bacteria in environments favored by chemosymbiotic hosts (27, 109). Another hypothesis is that because *Gammaproteobacteria* and *Alphaproteobacteria* fix carbon through

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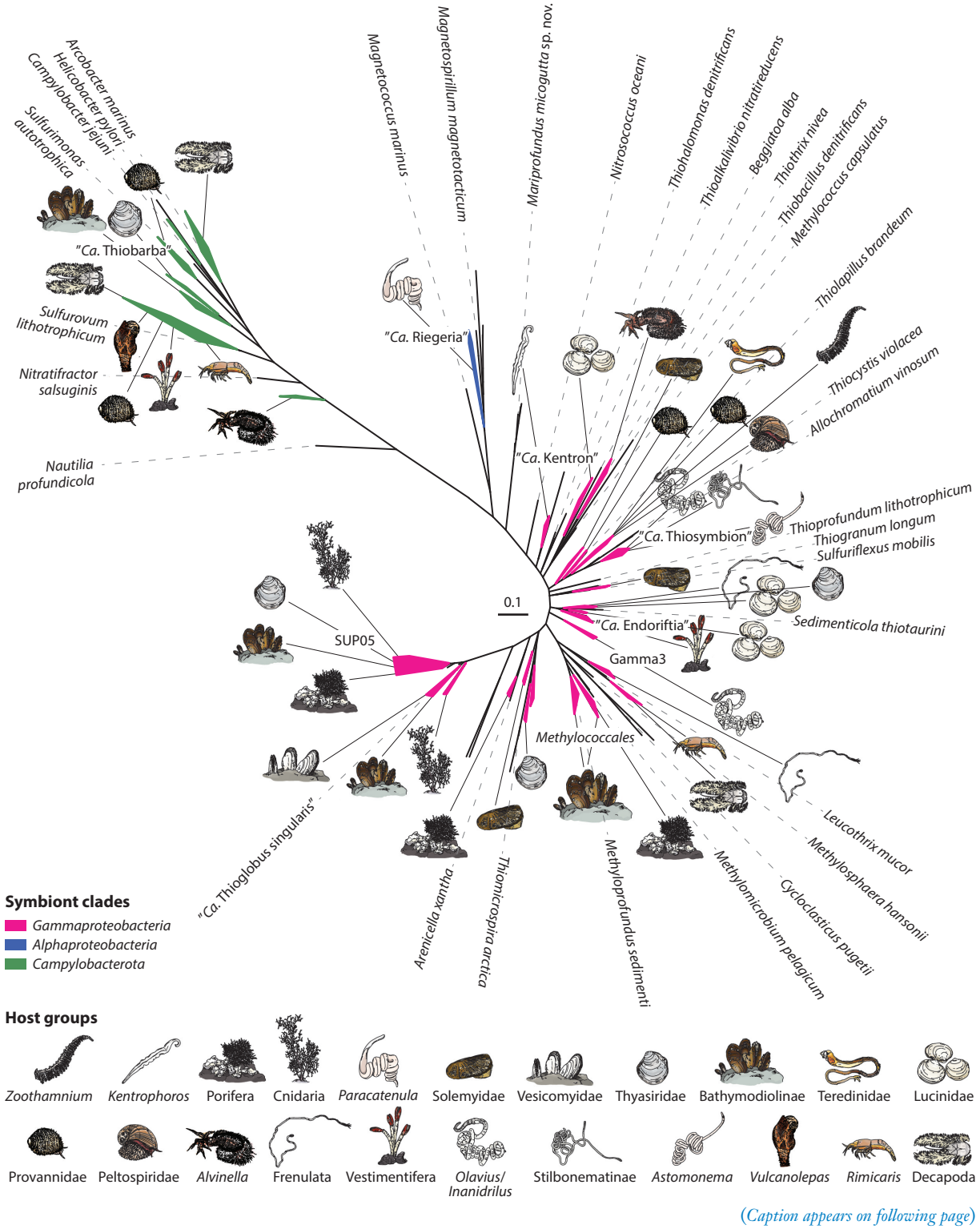

(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Phylogenetic diversity of chemosymbionts. Maximum likelihood tree of chemosymbionts based on their 16S rRNA genes. Major symbiont lineages are colored according to the bacterial group they belong to. Host groups are shown as icons. Solid lines indicate chemosymbioses highlighted in the review, and dashed lines point to selected and cultivated free-living relatives of symbionts. For the expanded 16S rRNA tree and methods see **Supplemental Figure 1**. Host group icons copyright Alina Esken (**https://www.alinaesken.de**).

Supplemental Material >

the Calvin-Benson-Bassham (CBB) cycle, they are likely to be best equipped for carbon fixation in most chemosynthetic environments (see Section 3.3). A third hypothesis is that many members of *Gammaproteobacteria*, *Alphaproteobacteria*, and *Campylobacterota* are preadapted to symbioses with eukaryotes but the genes involved and their distribution across symbiotic and free-living bacteria have yet to be described. None of these hypotheses have been systematically explored, highlighting the need for in-depth, comparative analyses of symbiotic and free-living chemosynthetic bacteria to better understand the processes that promote the establishment of these symbioses.

3. METABOLIC VERSATILITY OF CHEMOSYMBIONTS

The first step in chemosynthesis is the oxidation of reduced inorganic substrates or methane to generate energy and reducing equivalents. This energy powers carbon fixation or assimilation (**Figure 2**). While there are many ways chemosynthetic symbionts can perform chemosynthesis, the core central pathways for gaining energy from sulfide and methane, and for fixing carbon dioxide, are conserved across most symbionts (7, 63).

3.1. Energy Sources and Electron Donors

The vast majority of chemosynthetic symbionts are thiotrophs that oxidize reduced sulfur compounds to gain energy and reducing equivalents (**Figure 2*b***). Despite the fact that the free-living relatives of thiotrophic symbionts use several pathways for oxidizing reduced sulfur compounds, most chemosynthetic symbionts (alphaproteobacteria and gammaproteobacteria) use a similar combination of enzymes and pathways. These include sulfide quinone reductase (Sqr), flavocytochrome *c* (Fcc), a partial periplasmic sulfur oxidation enzyme complex (Sox), dissimilatory sulfite reductase (Dsr), APS reductase, and ATP sulfurylase. Together, these enzymes enable the oxidation of sulfide or thiosulfate to generate ATP and reducing equivalents needed for carbon fixation (63). The fact that the sulfur oxidation pathways of chemosymbionts from two classes and many clades within these classes have independently converged (3, 60) suggests that there is a selective advantage for thiotrophic symbionts in using these pathways to generate energy from reduced sulfur compounds. However, it should be noted that this convergence is not universal: "*Candidatus* Thiobarba," a campylobacterotal epibiont of bathymodiolin mussels, has a complete set of Sox genes and thus diverges from other known thiotrophic symbionts (3).

Methanotrophs are the second-most common type of chemosymbionts. These aerobic methane oxidizers use methane as an energy source as well as a carbon source (**Figure 2**). The presence of methanotrophs in a given host species is largely attributable to the availability of methane in the environment, not phylogeny. Thus, some bathymodiolin mussel species have methanotrophic symbionts, while their close relatives host only thiotrophic symbionts, depending on the concentrations and flux of methane and sulfide in the environment (101). Similarly, for host species in which methanotrophic and thiotrophic symbionts co-occur within the same individual, such as many species of bathymodiolin mussels, the relative abundance of these two symbionts is linked to the availability of sulfide and methane in their habitat (23, 50, 77, 89).

The central metabolic pathways of methanotrophic symbionts are known for only two host groups, bathymodiolin mussels and deep-sea sponges. Despite the large phylogenetic distance

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Table 2 Known chemosymbiont-host associations

Host phylum (WoRMS)	Host class (WoRMS)	Host family (WoRMS)	Host genus	Chemosymbionts	Energy source(s)	Carbon fixation pathway	Reference
Annelida	Clitellata	Naididae	*Inanidrilus*	Gammaproteobacteria	H_2S	CBB	9
			Olavius	Gammaproteobacteria, "*Candidatus* Thiosymbion"	H_2S, CO	CBB	65
			Tubificoides	Gammaproteobacteria	H_2S	CBB	108
	Polychaeta	Sabellidae	*Bispira*	Gammaproteobacteria	CH_4	RuMP (not confirmed)	48
		Serpulidae	*Laminatubus*	Gammaproteobacteria	CH_4	RuMP (not confirmed)	48
		Siboglinidae	*Siboglinum*	Gammaproteobacteria	H_2S	CBB (not confirmed)	123
			Escarpia	Gammaproteobacteria	H_2S	CBB, rTCA	73
			Galathealinum	Gammaproteobacteria	H_2S	CBB, rTCA	73
			Lamellibrachia	Gammaproteobacteria	H_2S	CBB, rTCA	73
			Oasisia	Gammaproteobacteria	H_2S	CBB	87
			Ridgeia	Gammaproteobacteria, *Campylobacterota* spp.	H_2S	CBB (both γ and ε), rTCA (ε, not confirmed)	107 42
			Riftia	Gammaproteobacteria, "*Candidatus* Endoriftia persephone"	H_2S	CBB, rTCA	102
			Sclerolinum	Gammaproteobacteria	H_2S	CBB (not confirmed)	37
			Seepiophila	Gammaproteobacteria	H_2S	CBB	73
			Tevnia	Gammaproteobacteria	H_2S	CBB, rTCA	43
		Alvinellidae	*Alvinella*	*Campylobacterota* spp.	H_2S	rTCA	14
Arthropoda	Thecostraca	Neolepadidae	*Vulcanolepas*	*Campylobacterota* spp.	H_2S	CBB (not confirmed)	121
	Malacostraca	Alvinocarididae	*Rimicaris*	Gammaproteobacteria, *Campylobacterota* spp.	H_2S	CBB, rTCA	61
		Kiwaidae	*Kiwa*	Gammaproteobacteria, *Campylobacterota* spp.	H_2S	CBB (not confirmed), rTCA (not confirmed)	47
		Munidopsidae	*Shinkaia*	Gammaproteobacteria, *Campylobacterota* spp.	H_2S, CH_4	CBB (not confirmed), *pmoA* amplified, rTCA (not confirmed)	128, 129

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Table 2 (*Continued*)

Host phylum (WoRMS)	Host class (WoRMS)	Host family (WoRMS)	Host genus	Chemosymbionts	Energy source(s)	Carbon fixation pathway	Reference
Cnidaria	Anthozoa	Plexauridae	*Paramuricea*	Gammaproteobacteria	H_2S	CBB	127
Mollusca	Bivalvia	Mytilidae	*Bathymodiolus*	Gammaproteobacteria	H_2S, CH_4, H_2	CBB, RuMP	96, 97
			Benthomodiolus	Gammaproteobacteria	H_2S	CBB (not confirmed)	
			Gigantidas	Gammaproteobacteria	H_2S, CH_4	CBB (not confirmed), RuMP	133
			Idas	Gammaproteobacteria	H_2S, CH_4	CBB, RuMP	33
			Vulcanidas	Gammaproteobacteria	H_2S	CBB (not confirmed)	132
		Lucinidae	*Codakia*	Gammaproteobacteria	H_2S	CBB	66
			Loripes	Gammaproteobacteria	H_2S	CBB	94
			Phacoides	Gammaproteobacteria	H_2S	CBB	74
			Lucina	Gammaproteobacteria	H_2S	CBB (not confirmed)	35
			Pegophysema	Gammaproteobacteria	H_2S	CBB (not confirmed)	28
			Lucinoma	Gammaproteobacteria	H_2S	CBB (not confirmed)	29
		Solemyidae	*Acharax*	Gammaproteobacteria	H_2S	CBB (not confirmed)	58
			Solemya	Gammaproteobacteria	H_2S	CBB	30
		Teredinidae	*Kuphus*	Gammaproteobacteria	H_2S	CBB	1
		Thyasiridae	*Conchocele*	Gammaproteobacteria	H_2S	CBB (not confirmed)	58
			Maorithyas	Gammaproteobacteria	H_2S	CBB (not confirmed)	135
			Thyasira	Gammaproteobacteria	H_2S	CBB	78
		Vesicomyidae	*Archivesica*	Gammaproteobacteria	H_2S	CBB	59
			Calyptogena	Gammaproteobacteria	H_2S	CBB (not confirmed)	91
			Phreagena	Gammaproteobacteria	H_2S	CBB	69
			Turneroconcha	Gammaproteobacteria	H_2S	CBB	88
	Gastropoda	Lepetodrilidae	*Lepetodrilus*	Gammaproteobacteria	H_2S	CBB (not confirmed)	4
		Neomphalidae	*Cyathermia*	*Campylobacterota* spp.	H_2S	rTCA	136
		Peltospiridae	*Chrysomallon*	Gammaproteobacteria	H_2S	CBB	85
		Provannidae	*Alviniconcha*	Gammaproteobacteria, *Campylobacterota* spp.	H_2S	CBB, rTCA	7 81
			Ifremeria	Gammaproteobacteria	H_2S	CBB, RuMP (not confirmed)	7, 54

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Table 2 (*Continued*)

Host phylum (WoRMS)	Host class (WoRMS)	Host family (WoRMS)	Host genus	Chemosymbionts	Energy source(s)	Carbon fixation pathway	Reference
Nematoda	Chromadorea	Siphonolaimidae	*Astomonema*	Gammaproteobacteria	H_2S	CBB (not confirmed)	83
		Desmodoridae	*Laxus*	Gammaproteobacteria	H_2S	CBB	94
			Leptonemella	Gammaproteobacteria	H_2S	CBB	94
			Robbea	Gammaproteobacteria	H_2S	CBB	92
Platyhelminthes	Catenulida	Retronectidae	*Paracatenula*	Alphaproteobacteria	H_2S	CBB	49
Porifera	Demospongiae	Hymedesmiidae	*Hymedesmia*	Gammaproteobacteria	CH_4, H_2S	EMP variant of RuMP pathway, CBB	106
		Acarnidae	*Iophon*	Gammaproteobacteria	CH_4	EMP variant of RuMP pathway	106
		Cladorhizidae	*Cladorhiza*	Gammaproteobacteria	CH_4	RuMP (not confirmed)	54
		Irciniidae	*Ircinia*	Gammaproteobacteria	H_2S	CBB, rTCA, HB-HB (from metagenomic bins)	38
Ciliophora	Karyorelictea	Kentrophoridae	*Kentrophoros*	Gammaproteobacteria	H_2S	None, obligate heterotroph	119
	Oligohymenophorea	Zoothamniidae	*Zoothamnium*	Gammaproteobacteria	H_2S	CBB	100
Euglenozoa	Euglenoidea	Euglenaceae	*Calkinsia*	*Campylobacterota* spp.	H_2S	rTCA (not confirmed)	36

Abbreviations: CBB, Calvin-Benson-Bassham cycle; EMP, Embden-Meyerhof-Parnas pathway; HB-HB, 3-hydroxypropionate/4-hydroxybutyrate cycle; rTCA, reductive tricarboxylic acid cycle; RuMP, ribulose monophosphate pathway; WoRMS, World Register of Marine Species.

between sponges and mussels, their symbionts use similar pathways for methane assimilation (97, 106). The symbionts oxidize methane to methanol through a monooxygenase complex (PmoCAB) and further to formaldehyde using a methanol dehydrogenase (XoxF). The formaldehyde is transported into the symbiont's cytoplasm, where it is either used to build biomass through variants of the ribulose monophosphate pathway or further oxidized to formate to

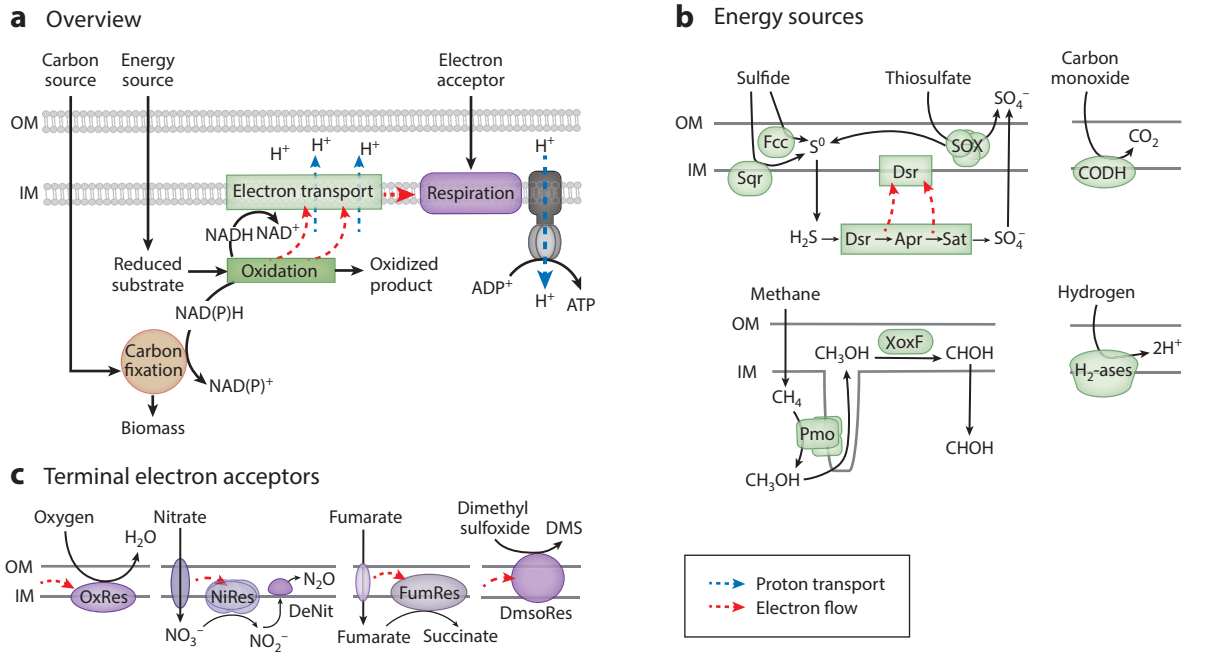

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Figure 2 (*Figure appears on preceding page*)

An overview of the metabolic pathways chemosynthetic symbionts use to gain energy and build biomass. (*a*) A schematic outlining the general processes involved in chemosynthesis, including the flow of electrons, hydrogen ion transport, and the generation of ATP and reducing equivalents (NAD^+) to power carbon fixation. (*b*) Chemosynthetic symbionts use diverse energy sources to power biosynthesis. Reduced sulfur compounds (sulfide, thiosulfate), carbon monoxide, and hydrogen have been shown to be used by thiotrophs, while methanotrophs are only known to use methane. (*c*) Omics evidence indicates chemosynthetic symbionts use oxygen, nitrate, fumarate (and other organic acids), and dimethyl sulfoxide as terminal electron acceptors. (*d*) Chemosynthetic symbionts fall along a spectrum: from obligate autotrophy, with a metabolism optimized for inorganic carbon fixation, to obligate heterotrophy, requiring organic carbon sources. Most symbionts are mixotrophs. Key enzymes involved in acquisition of energy are in green, respiration in purple, and carbon assimilation in brown. Abbreviations: ACL, ATP-citrate lyase; ACS, acetyl-CoA-synthase; Apr, APS reductase; CBB, Calvin-Benson-Bassham cycle; CODH, CO dehydrogenase; C_{org}, organic carbon; DeNit, denitrification; DMS, dimethyl sulfide; DmsoRes, dimethyl sulfoxide respiration; Dsr, dissimilatory sulfite reductase; Fcc, flavocytochrome *c*; FumRes, fumarate respiration; H_2-ase, hydrogenase; H_4F, tetrahydrofolate pathway; H_4MPT, dephospho-tetrahydromethanopterin; IM, inner membrane; MclA, malyl-CoA lyase; NiRes, nitrate respiration; OM, outer membrane; OxRes, oxygen respiration; PEP, phosphoenolpyruvate; Pmo, particulate methane monooxygenase complex; rTCA, reductive tricarboxylic acid pathway; RuMP, ribulose monophosphate pathway; SagA, serine-glyoxylate aminotransferase; Sat, ATP sulfurylase; SOX, sulfur oxidation enzyme complex; Sqr, sulfide:quinone oxidoreductase; TCA, tricarboxylic acid cycle; XoxF, methanol dehydrogenase.

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generate NADPH and carbon dioxide (15, 16, 20, 106). This energy-generating step is accomplished in parallel by the tetrahydrofolate and tetrahydromethanopterin pathways and is predicted to act as an overflow mechanism to avoid the buildup of formaldehyde, which is toxic to cellular metabolism (24, 97, 106).

Only a small number of methanotrophic symbionts have been characterized genomically, but many uncharacterized methanotrophs are associated with tube worms, *Ifremeria* snails, feather duster worms, and colonial ciliates (10, 48, 90, 103). Comparative analyses are needed to reveal whether the pathways of methanotrophic symbionts have converged in a similar manner as in thiotrophic symbionts. If so, this would suggest that symbiosis constrains the metabolism of chemosynthetic symbionts in ways not experienced by free-living chemosynthetic bacteria.

For over 35 years after the discovery of chemosynthetic symbioses, reduced sulfur compounds and methane were the only energy sources known to fuel these associations. The ability of chemosymbionts to use hydrogen as an energy source was first discovered in 2011, in the thiotrophic symbionts of *Bathymodiolus* mussels (85, 96). However, the hydrogenase genes needed to oxidize hydrogen are not present in all thiotrophic symbionts of *Bathymodiolus* mussels. They are strain-specific and vary with the availability of hydrogen in the environment (2, 57). The thiotrophic symbionts of the tube worm *Riftia* also express hydrogenase genes, but they do not appear to use hydrogen as an electron donor. This highlights a fact not always fully acknowledged in recent studies, that metagenomic analyses alone are not sufficient for interpreting the metabolism of microorganisms (80).

As with hydrogen, carbon monoxide was well known to fuel the metabolism of free-living bacteria but was only recently discovered to play a role in chemosynthetic symbioses, specifically, in the sulfur-oxidizing and sulfate-reducing symbionts of the gutless oligochaete *Olavius algarvensis* (64, 65). Intriguingly, the discovery of genes for carbon monoxide oxidation in the *O. algarvensis* symbionts led to the realization that the dead seagrass rhizomes in these worms' environment are the likely source of carbon monoxide. This highlights the value of studying chemosynthetic symbioses for environmental microbiology: These high-abundance, low-diversity microbial communities are easier to analyze using meta-omics than many free-living microbial communities with high diversity but low abundances of individual species. Chemosymbioses can thus alert researchers to the availability of energy and carbon sources not previously considered to play a role in a given environment.

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3.2. The Electron Acceptors

All animal hosts and most chemosynthetic symbionts use oxygen as the terminal electron acceptor (TEA) during cellular respiration (**Figure 2*c***). In some symbioses, the oxygen consumption rates are so high that oxygen may be the most limiting factor for host and symbiont metabolism. The high oxygen demands of chemosynthetic symbionts place a cost on their hosts, and these have evolved a range of adaptations to meet the aerobic demands of their symbionts (reviewed in 21, 116).

Because oxygen is not uniformly distributed within or across chemosynthetic habitats, hosts must ensure that their symbionts receive enough oxidants and reductants to meet their metabolic demands. For example, the tube worm *Riftia* has a modified hemoglobin with a single–amino acid substitution that allows it to bind both sulfide and oxygen (**Figure 3**). This adaptation ensures that both substrates are delivered to the symbionts deep inside the tube worm's body (40). Shallow-water chemosynthetic hosts, such as gutless oligochaetes, nematodes, flatworms, and ciliates, migrate between oxic and anoxic sediment layers, thus providing their symbionts the oxidants and reductants needed for cellular respiration and chemosynthesis (e.g., 45).

Genomic, proteomic, and physiological evidence indicates that many chemosynthetic symbionts also use other TEAs besides oxygen (**Figure 2*c***). Nitrate is often available in the upper layers of marine sediments and can be used as a TEA by the thiotrophic symbionts of lucinid clams (53), oligochaetes, and nematodes (65, 94, 134). The "*Candidatus* Thiosymbion" thiotrophs of oligochaetes and nematodes may also use organic acids and dimethyl sulfoxide as oxidants during cellular respiration (65, 94, 134). The ability to respire these alternative TEAs that eukaryotes cannot use prevents competition of the symbionts with their hosts for oxygen. Niche partitioning for TEAs has also been observed among co-occurring thiotrophic symbionts. For example, *O. algarvensis* houses two types of thiotrophic symbionts, "*Ca.* Thiosymbion," which uses oxygen as a TEA, and a second gammaproteobacterial symbiont, Gamma3, which lacks the genes necessary to use oxygen as a TEA and instead has the genes for respiring nitrate (65, 94, 134).

3.3. Inorganic Carbon Fixation

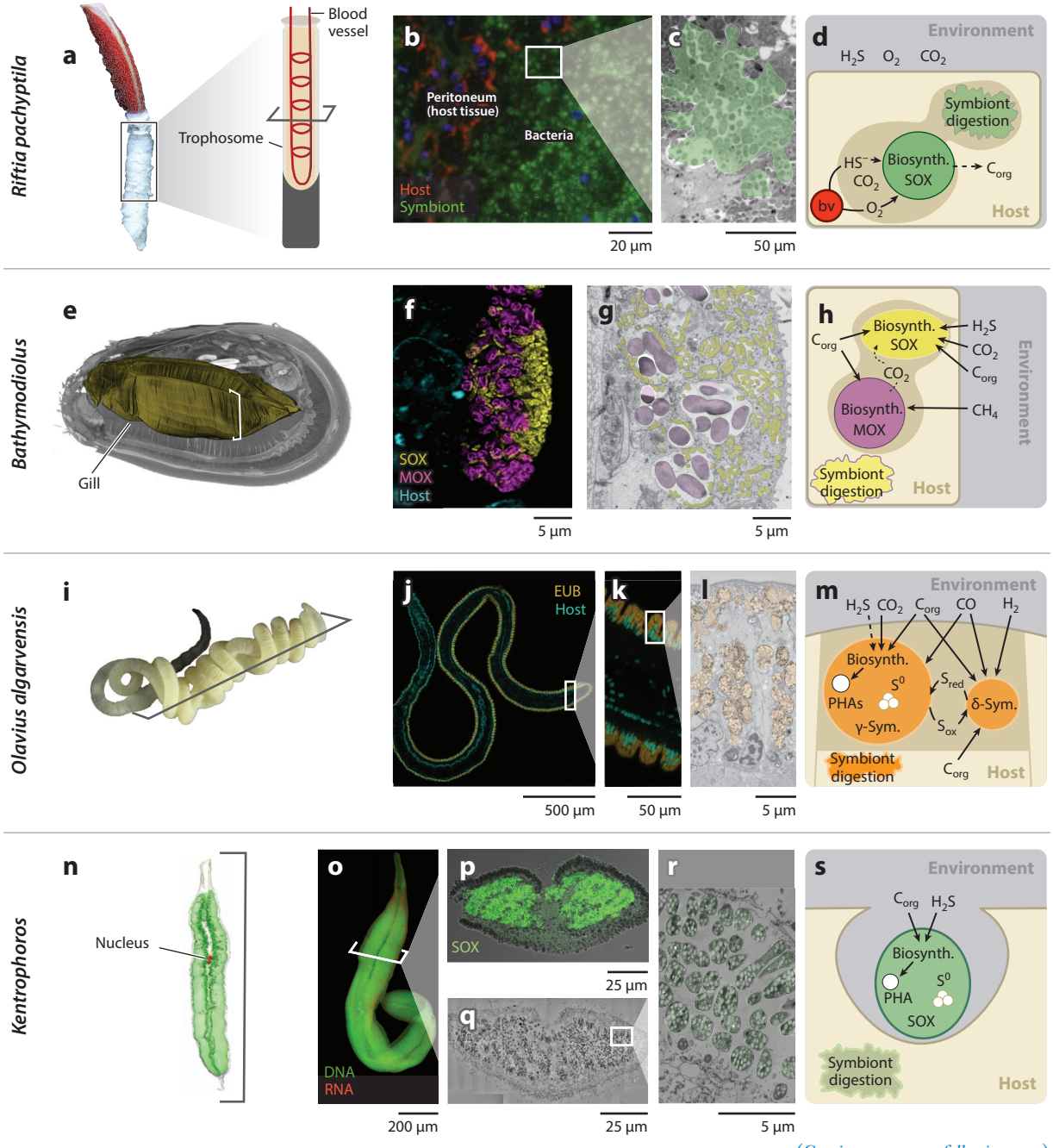
Seven pathways for fixing carbon dioxide into biomass have now been described (56, 110). Three of these have been described as being from thiotrophic symbionts: (*a*) a modified version of the CBB cycle, (*b*) the reductive tricarboxylic acid (rTCA) cycle, and (*c*) the Wood-Ljungdahl pathway (reductive acetyl-CoA pathway) (e.g., 18, 65, 76, 88) (**Figure 2*d***).

The CBB cycle is by far the dominant pathway for carbon fixation on Earth (56) and the most common pathway in thiotrophic symbionts (**Table 2**). The CBB cycle in most thiotrophic symbionts and many free-living chemoautotrophs is modified from the textbook version of the CBB cycle for higher energy efficiency. The classical CBB enzymes fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphophase are replaced by a reversible and inorganic pyrophosphate–dependent 6-phosphofructokinase (PPi-PFK). This modification saves approximately 9% energy during carbon fixation in comparison to the canonical CBB cycle, thus providing bacteria that use the modified version a selective advantage in energy-limited environments (30, 60, 63, 76).

Gammaproteobacterial thiotrophs of tube worms and the campylobacterotal thiotrophs of deep-sea shrimp, gastropods, and polychaetes use the rTCA cycle for carbon fixation (8, 14, 73, 76, 120). As the name implies, the rTCA cycle reverses the oxidative TCA pathway to produce acetyl-CoA from two molecules of carbon dioxide at the expense of 2 ATP and 2 reducing equivalents. While the rTCA cycle is energetically less costly than the CBB cycle, some of the enzymes are sensitive to the presence of oxygen. It is therefore assumed that chemoautotrophs from anaerobic or microaerobic habitats preferentially use the rTCA cycle over the CBB (56).

However, some chemosynthetic symbionts, like those of the deep-sea snail *Alviniconcha*, use the rTCA cycle regardless of the concentration of oxygen in their environment (8).

These two carbon fixation pathways, the oxygen-sensitive rTCA and oxygen-tolerant CBB cycle, tend to be phylogenetically distributed. The *Gammaproteobacteria*, which typically dominate more oxic chemosynthetic habitats, commonly use the CBB cycle for carbon fixation (56), while



(*Caption appears on following page*)

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Figure 3 (*Figure appears on preceding page*)

Examples of chemosynthetic symbioses, highlighting their diversity in body plans and carbon acquisition strategies. (*a–d*) The giant tube worm *Riftia pachyptila* houses its endosymbionts (*green*) in an internal organ called the trophosome. (*b*) Image from FISH with a probe specific to the symbionts and (*c*) a TEM image. (*d*) *Riftia* has a modified hemoglobin that delivers both sulfide (H_2S) and oxygen to its SOXs through its blood vessels. The symbionts fix carbon dioxide into biomass that is delivered to the host primarily through intracellular symbiont digestion. (*e–h*) The deep-sea mussel *Bathymodiolus* from the Mid-Atlantic Ridge, illustrated here with micro–computed tomography, houses its SOXs and MOXs in its gill tissues. (*f*) FISH image of a mussel gill cell (bacteriocyte) with a probe specific to the MOXs (*purple*) and SOXs (*yellow*). (*g*) TEM image of a bacteriocyte with co-occurring MOXs (*purple*) and SOXs (*yellow*). (*h*) By pumping seawater across its gills, the mussel provides its symbionts with reductants (reduced sulfur compounds for SOXs, methane for MOXs), carbon (carbon dioxide for SOXs, methane for MOXs), and oxygen. The end product of methane oxidation by the MOXs, carbon dioxide, may be fixed by the SOXs, which also fix carbon dioxide from the seawater. (*i–m*) The gutless oligochaete *Olavius* lacks a digestive system (mouth, gut, anus) and excretory system and relies on its symbionts for both its nutrition and waste management. The worm harbors its symbionts directly underneath the outer layer (cuticle) of its body wall. (*j*,*k*) FISH images of a longitudinal section through the worm showing the symbionts on both sides of the worm's body wall with a general eubacterial probe (*yellow*) and host tissues (DAPI staining, *green*). (*l*) TEM image of symbiotic bacteria directly below the worm's cuticle and above its epidermal cells. (*m*) Syntrophic sulfur cycling of reduced sulfur compounds produced by deltaproteobacterial sulfate-reducing symbionts and oxidized sulfur compounds produced by gammaproteobacterial SOXs maximizes energy gain. Sulfur and PHAs are stored inside symbiont cells as energy and carbon reserves. (*n–s*) The single-celled, sediment-dwelling ciliate *Kentrophoros* has epibiotic SOXs on the outside of its body, as shown by (*o*) acridine orange staining (*green*, DNA; *red*, RNA), (*p*) FISH with a "*Candidatus* Kentron"–specific probe, and (*q*,*r*) TEM. (*s*) Metabolic reconstruction shows "*Ca.* Kentron" uses reduced sulfur compounds to produce biomass from highly-oxidized organic carbon, e.g., carboxylates. Abbreviations: bv, blood vessel; C_{org}, organic carbon; δ-sym., deltaproteobacterial symbiont; EUB, eubacterial probe; FISH, fluorescence in situ hybridization; γ-sym., gammaproteobacterial symbiont; MOX, methane-oxidizing symbiont; PHA, polyhydroxyalkanoate; S^0, elemental sulfur; S_{ox}, oxidized sulfur; SOX, sulfur-oxidizing symbiont; S_{red}, reduced sulfur compounds; TEM, transmission electron microscopy. Panel *b* adapted with permission from Reference 113. Panel *c* adapted with permission from Reference 13. Panel *e* adapted from Reference 44. Panel *f* provided by M. Franke. Panel *g* provided by N. Leisch. Panels *i–l* provided by A. Gruhl. Panel *n* adapted with permission from Reference 117. Panels *o* and *p* provided by B. Seah. Panels *q* and *r* adapted from Reference 118.

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the *Campylobacterota*, which dominate lower-oxygen habitats, tend to use the rTCA cycle (79). One notable exception was recently described. "*Ca.* Thiobarba," the campylobacterotal epibiont of bathymodiolin mussels, lacks the genes for the rTCA cycle and instead uses the CBB cycle (3). These symbionts gained the genes for the CBB pathway through multiple events of horizontal gene transfer, suggesting that their association with mussels induced selective pressure to use a more oxygen-tolerant pathway for carbon fixation. As another example of symbiont adaptation to maximize carbon fixation, the thiotrophic symbionts of some tube worms express the genes for both the CBB and rTCA pathways (73, 76, 107). These symbionts may be able to selectively use the carbon fixation pathway most efficient for the redox conditions in their highly variable environments. How the use of these two pathways is regulated is an interesting question for future research.

The third pathway for carbon dioxide fixation, the reductive acetyl-CoA pathway, consists of a set of reversible reactions that convert two molecules of carbon dioxide into acetyl-CoA using carbon monoxide dehydrogenase and acetyl-CoA synthase. However, because the enzymes work in both the reductive and oxidative directions, it is unclear whether chemosynthetic symbionts use these genes for autotrophic carbon dioxide fixation or heterotrophic carbon assimilation (**Figure 2**) (6, 62, 65). Furthermore, the acetyl-CoA pathway has been found only in bacteria whose chemosynthetic nature has not been proven, such as the deltaproteobacterial symbionts of ciliate shrimp and oligochaetes (see the sidebar titled Putative Chemosymbionts).

3.4. The Autotrophic-Heterotrophic Spectrum of Carbon Acquisition

Very few thiotrophic symbionts appear to be obligate autotrophs. The thiotrophic symbionts of vesicomyid clams may be the only ones: These lack the TCA cycle gene α-ketoglutarate

dehydrogenase, a condition thought to indicate obligate autotrophy (88). One intriguing hypothesis for the selective advantage of obligate autotrophy is that genome reduction in these vertically transmitted symbionts led to the loss of genes needed for heterotrophy.

On the other end of the spectrum lies obligate heterotrophy, but researchers long assumed that autotrophic fixation of carbon dioxide is intrinsic for thiotrophic symbioses. The recent discovery of sulfur-oxidizing symbionts in *Kentrophoros* ciliates that lack the canonical genes for autotrophic carbon fixation led to a paradigm shift in our understanding of these associations (119). The ciliate symbionts use the energy gained from oxidizing sulfide to fuel the uptake of organic compounds from the environment. They are the only known chemosymbionts that are obligate heterotrophs (119).

The vast majority of chemosynthetic symbionts appear to be mixotrophs that use both inorganic and organic sources of carbon (e.g., 30) (**Figure 3*d*,*h*,*m*,*s***). Genomic signatures of heterotrophic metabolism include the presence and expression of genes for a complete TCA cycle, genes involved in glycolysis, and transporters for the uptake of a variable range of organic compounds (65, 74, 98, 99, 102). Some symbionts have pathways for assimilating organic waste compounds, such as acetate and propionate, that their hosts produce under anaerobic conditions. For example, symbionts of *O. algarvensis* and the flatworm *Paracatenula* accomplish this using a modified version of the 3-hydroxypropionate bicycle (60, 65) (**Figure 3*m***). The selective advantages of a mixotrophic lifestyle include reducing carbon loss from the symbiosis by recycling host waste products and using organic compounds as electron sinks for oxidative pathways (65, 119). The degree to which heterotrophic carbon contributes to the net growth of symbionts and hosts is, however, not clear. This is because it is challenging to distinguish between the organic carbon assimilated by the symbionts from the environment and the internal carbon they recycle from the host's waste products.

3.5. Nutritional Transfer

The transfer of nutrients from chemosynthetic symbionts to their hosts is thought to occur via three modes (**Figure 3*d*,*h*,*m*,*s***). Symbiont digestion is the first and most common mode of nutrient transfer and occurs in endosymbiotic associations through phagolysosomal digestion of the symbionts inside host cells. Intracellular symbiont digestion has been observed in many hosts, including mussels, clams, tube worms, and oligochaetes (12, 67, 97, 134). Some hosts with epibiotic bacteria, such as the vent crab *Shinkaia*, may graze on their symbionts with their mouthparts and then digest them in their guts (129).

Alternatively, hosts can milk their symbionts. Milking, or the direct transfer of organic carbon in the form of small molecules, such as sugars and amino acids, from the symbiont to the host, is a common form of nutrient exchange in many algal-invertebrate symbioses (52, 84). Milking may play a role in some hosts with thiotrophic ectosymbionts, such as *Rimicaris* and *Shinkaia* (98, 128). Milking is also hypothesized to play a role in *Riftia* endosymbiosis, particularly during periods of high symbiont productivity, but its contribution is likely minor given convincing morphological and proteomic evidence for intracellular symbiont digestion (55). We hypothesize that milking plays at best only a small role in chemosymbioses, as the amount and type of nutrients that can be transferred to the host are substantially limited in this form of nutritional transfer.

Recently, researchers proposed a third mode of nutrient transfer via the bacterial release of outer membrane vesicles (60). Outer membrane vesicles serve a number of roles and are predicted to facilitate nutrient transfer between both free-living members of microbial communities and symbiotic partners (reviewed elsewhere; e.g., 115). Symbiont secretion of outer membrane vesicles would be advantageous in chemosynthetic associations because it would allow the direct transfer of a broad range of nutrients, such as lipids, proteins, sugars, amino acids, and nucleic acids (19).

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One issue in quantifying the contribution of symbiont-fixed carbon to the host is that anaplerotic carbon fixation is rarely considered in studies measuring the transfer of nutrition in chemosynthetic symbioses. Anaplerotic carbon fixation refers to the fixation of inorganic carbon by carboxylases in central carbon metabolism and is common to all animals and most microorganisms (for an overview see 68). Anaplerosis can contribute up to a third of an organism's carbon content and accounts for up to 10% of total cell carbon in bacteria (93, 105, 114). This highlights the importance of appropriate controls in physiological experiments to distinguish between carbon fixed by the host and carbon fixed by the symbionts and transferred to the host.

3.6. Acquisition of Other Nutrients

The availability of essential nutrients such as nitrogen and phosphorus often limits marine microbial productivity in open ocean waters. For most chemosymbioses, nitrogen may not be limiting, as organic and inorganic nitrogen are readily available in many shallow-water and deep-sea environments. All symbionts sequenced thus far have pathways to assimilate ammonium and/or nitrate (46, 70, 72, 111). Additionally, many symbionts are efficient in recycling host nitrogenous compounds such as osmolytes and waste compounds like glycine betaine and urea (65, 71, 72). The ability of chemosymbionts to fix nitrogen directly, although long hypothesized, was only recently discovered in the sulfur-oxidizing symbionts of lucinid clams and nematodes (66, 94). Given that nitrogen fixation is costly, chemosynthetic symbionts likely use this strategy only when nitrogenous compounds in the environment are limiting.

Our understanding of nitrogen metabolism in chemosynthetic symbioses is still shallow, but even less is understood about phosphorus acquisition. Dissolved inorganic phosphate is likely patchily distributed across hydrothermal vents (22, 39). It is therefore intriguing that the thiotrophic symbionts of some bathymodiolin mussels have differentially retained the genes needed to acquire phosphate from the environment when this essential nutrient is present at low concentrations, specifically a high-affinity phosphate transport system (PstSCAB) and a two-component regulatory system (PhoR-PhoB) (2). This suggests that at some sites where phosphate may be scarce, the ability to sense and acquire phosphate is important in the maintenance of the symbiosis (2).

3.7. Nonnutritional Symbiont Functions

Given that the majority of research in chemosynthetic symbioses has centered on describing and understanding nutritional interactions, it is not surprising that researchers understand less about other symbiont services. Emerging evidence indicates some chemosynthetic symbionts may also provide protective services. The sulfur-oxidizing symbionts of bathymodiolin mussels have and express unusually large numbers of toxin-related genes, many of which are predicted to encode proteins similar to toxins of highly pathogenic bacteria and to insecticidal toxins of terrestrial invertebrates. These are hypothesized to protect the mussels against pathogens and parasites (112).

3.8. Stronger Together: Symbiont-Symbiont Interactions

In associations with multiple symbiont species, selection is predicted to favor interactions between the symbionts that benefit the host. One of the well-studied examples of beneficial symbiont-symbiont interactions is the syntrophic cycling of sulfur compounds between the symbionts of *O. algarvensis*. The habitat of these worms, seagrass sediments in the Mediterranean, have such low sulfide concentrations that it was unclear how the sulfur-oxidizing symbionts of these oligochaetes gain enough energy. Researchers discovered that these worms have sulfate-reducing bacteria that

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produce the reduced sulfur compounds that the sulfur-oxidizing bacteria need to gain energy (32, 134) (**Figure 3*m***). Cycling of oxidized and reduced sulfur compounds between the sulfur-oxidizing and sulfate-reducing symbionts is hypothesized to increase productivity, as shown for cocultures of these two types of bacteria (32).

Another example of beneficial interactions between chemosynthetic symbionts may be the recycling of carbon compounds. Past studies hypothesized that in bathymodiolin mussels that harbor co-occurring thiotrophic and methanotrophic symbionts, the carbon dioxide produced by the methanotrophs through the oxidation of methane is assimilated by the thiotrophs (86, 97). One advantage of this recycling could be that the thiotrophs would not be limited by carbon dioxide during periods when they encounter high concentrations of their energy sources. We predict that given the strong selective advantage that nutritional interactions between co-occurring symbionts provide to their hosts, these are much more common than currently described.

4. CURRENT CHALLENGES AND FUTURE DIRECTIONS

Advances in our understanding of chemosynthetic symbioses have been driven largely by the methods available to researchers. In the first decades after the discovery of chemosymbioses in 1977, ultrastructural and physiological studies laid the basis for describing the morphology of these partnerships and the processes that enable the symbionts to gain energy, carbon, and nutrients from the environment. In the last two decades, omics analyses revealed the remarkable phylogenetic diversity of chemosynthetic symbionts and provided a wealth of data for predicting how these nutritional symbioses function and how they evolved. The time is now ripe for an integrated approach that combines imaging, physiology, in situ and ex situ experiments, omics, and modeling to gain a comprehensive understanding of the ecological and evolutionary processes that have shaped the remarkable success of chemosynthetic symbioses. In the following, we highlight a few approaches that would help move the field forward.

There is a clear need to develop metabolic models to better describe and quantify the contributions and costs of the partners in these nutritional associations and their impact on ecosystem-level processes (5). In other symbiotic systems, for example coral-algal symbioses, dynamic energy budget models are widely applied to explore the balance between autotrophic and heterotrophic metabolisms (e.g., 25). To properly apply these models to chemosynthetic systems, it will be critical to collect data across ecosystem to cellular scales, ranging from environmental data on the availability of oxidants, reductants, and carbon to in situ biochemical and physiological data on rates of assimilation, respiration, and growth.

How homeostasis is maintained in chemosynthetic symbioses is another question worth pursuing. There is clearly a fine-tuned balance between symbiont biomass and digestion, but nothing is known about how this steady state is maintained. Is it driven by the host, the symbiont, or both, and are the processes involved in maintaining this balance between symbiont growth and host digestion similar across chemosymbiotic associations? Symbiont cultivation and genetic manipulation would help answer these questions but have not yet been achieved. An alternative approach is targeted genome editing of the host using tools that are now widely available, such as CRISPR/Cas and RNA interference.

The role of bacteriophages in the physiology and metabolism of chemosynthetic symbioses has not yet been studied. Chemosynthetic symbionts, which occur as monocultures, or low-diversity polycultures, should be highly susceptible to phage infection, which would considerably diminish the symbiont population (122). There is, however, no evidence for phage-induced population collapses in chemosynthetic symbionts, and it remains unclear which mechanisms prevent phage predation on symbionts. Based on genomic evidence from some chemosynthetic symbionts,

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symbiont-phage interactions must occur at some point in the bacteria's life cycles. Specifically, the genomes of chemosynthetic symbionts associated with bathymodiolin mussels, cold-seep sponges, gutless oligochaetes, and tube worms encode a diversity of CRISPR systems that are responsible for defense against phages (11, 97, 124, 134). Future metagenomic studies of chemosymbiotic associations should include virome targets to allow us to better explore chemosymbiont-phage interactions.

5. CONCLUSIONS

The pronounced phylogenetic diversity of chemosynthetic symbioses reflects the strong selective advantage these associations provide to both eukaryotic hosts and chemosynthetic bacteria that live together. The marked metabolic diversity of chemosynthetic symbioses reflects the wealth of energy, carbon, and other nutrient sources available in marine environments and the adaptive ability of these associations to make use of favorable resources in their environment. We predict that we are far from discovering the full phylogenetic and metabolic diversity of chemosynthetic symbioses. As we reveal and describe the sweeping diversity of these symbiotic unions, we will eventually acquire the information needed for a comprehensive understanding of the ecological and evolutionary processes that have enabled the ubiquitous success of chemosynthetic associations.

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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