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Review

On the Origin of Heterotrophy

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The theory of autotrophic origins of life posits that the first cells on Earth satisfied their carbon needs from CO_2. At hydrothermal vents, spontaneous synthesis of methane via serpentinization links an energy metabolic reaction with a geochemical homologue. If the first cells were autotrophs, how did the first heterotrophs arise, and what was their substrate? We propose that cell mass roughly similar to the composition of *Escherichia coli* was the substrate for the first chemoorganoheterotrophs. Amino acid fermentations, pathways typical of anaerobic clostridia and common among anaerobic archaea, in addition to clostridial type purine fermentations, might have been the first forms of heterotrophic carbon and energy metabolism. Ribose was probably the first abundant sugar, and the archaeal type III RubisCO pathway of nucleoside monophosphate conversion to 3-phosphoglycerate might be a relic of ancient heterotrophy. Participation of chemiosmotic coupling and flavin-based electron bifurcation – a soluble energy coupling process – in clostridial amino acid and purine fermentations is consistent with an autotrophic origin of both metabolism and heterotrophy, as is the involvement of S^0 as an electron acceptor in the facilitated fermentations of anaerobic heterotrophic archaea.

Trends

The physiology of anaerobic autotrophs is rich in exergonic, H_2-dependent CO_2 reductions and transition metal catalysis, properties shared with spontaneous chemical reactions at hydrothermal vents. If the first cells were autotrophs, how did the first heterotrophs arise?

By dry weight, modern cells are made mostly of protein (~55%) and RNA (~25%). A diet of that type was, we propose, the carbon and energy source for the first heterotrophs.

Clostridial-type amino acid and purine fermentations as well as sulfur-dependent fermentations of anaerobic archaea might hold clues about the physiology of the first heterotrophs.

At the high H_2 partial pressures of vents, anaerobic amino acid synthesis is exergonic. Amino acid fermentations can thus only have arisen at low H_2 partial pressures, for example in an extinguished vent or in accumulated cell sediments.

Autotrophic Origins

Views on the earliest phases of evolution fall into two main camps: autotrophic origins vs. heterotrophic origins. Theories for autotrophic origins posit that the first cells satisfied their carbon needs from CO_2 [1,2] while heterotrophic origin theories have it that the first cells lived from the fermentations of reduced organic compounds present in some kind of rich organic soup [3]. The heteotrophic origin theory, while traditionally favored by chemists [4], has two main drawbacks. Seen from a biological perspective, it does not connect with the chemistry of modern cells, starting with cyanide [5], formamide [6], or UV-light-dependent condensations [7], chemicals and reactions that do not occur in microbial core carbon and energy metabolism. Nor does it connect well with either geology or geochemistry, requiring nonaqueous syntheses or other specific conditions such as prebiotic sugar phosphates in the early oceans that, despite appearing plausible to proponents [8], are unlikely to ever have existed on early Earth [9,10].

Autotrophic origin theories, by contrast, tend to operate with chemicals that undoubtedly did exist on the anaerobic early Earth: H_2, CO_2, N_2, and H_2S [11–14]. Transition metals and transition metal sulfide (FeS and FeNiS) centers [15,16] play a crucial role in autotrophic origin theories, for several reasons. They serve as catalysts in core carbon and energy metabolism of anaerobic autotrophs [17], they abound in anaerobic geological settings, both today and on the early Earth [12,13], and they are naturally catalytic by virtue of their unfilled *d* and *f* electron orbitals, which can readily hybridize to generate metastable bonds with carbon and nitrogen.

Modern anaerobic autotrophs thrive upon H_2, which is continuously generated in serpentinizing geological settings at activities of the order of 10 mmol kg^{-1} or more [18] via disequilibria driven by rock–water interactions in hydrothermal systems [19,20]. At such H_2 activities, and under strictly anaerobic conditions, the synthesis of cell mass from CO_2 is thermodynamically favorable

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[21]. The core pathway of carbon and energy metabolism in anaerobic autotrophs that inhabit such hydrothermal and deep crust environments [22–24] is the acetyl-coenzyme A (CoA) pathway, the most ancient of the six pathways of CO_2 fixation known and the only one present in archaea and bacteria [2,25]. Spontaneous exergonic organic syntheses from H_2 and CO_2 occur today at hydrothermal vents, for example formate synthesis [18,26] and methane synthesis [19,20,27], that are similar, if not homologous [28], to core energy-releasing reactions of carbon and energy metabolism in methanogens and acetogens, which live by the reduction of CO_2 by H_2. Microbial communities have been thriving in hydrothermal vents for over 3.3 billion years [29].

Isotope evidence indicates the antiquity of methanogenesis [30], and some new phylogenies even place the root of the archaea within the methanogens [31,32]. Moreover, the recent laboratory synthesis of formate, acetate, methanol, and pyruvate from CO_2 – using gregite (Fe_3S_4) as a catalyst under simulated alkaline hydrothermal vent conditions [33] in addition to the FeNiS-catalyzed synthesis of CO and formaldehyde [34] under simulated alkaline vent conditions – support a specific formulation of autotrophic origins that has methanogens and acetogens as the first forms of life. Given those circumstances, let us assume for a moment that the first cells really were autotrophs, from which a surprising question ensues: how did heterotrophs arise? Here we address the possible physiology of the first heterotrophs and the nature of the growth substrates for their fermentations.

Cells: Much Better than Stardust and Mostly Protein

A logical consequence of autotrophic origins is that anaerobic autotrophs were not only the ancestors of the first heterotrophs, they were also the first viable substrate for heterotrophic growth (Box 1). Critics might interject that there were huge amounts of reduced carbon compounds delivered to Earth from space [35], and that such material also could have served as viable substrate for heterotrophs. Organics from space unquestionably did accumulate on the early Earth, but could they have been a viable substrate? The answer is no. Organics delivered from space, despite comprising as much as perhaps 10% of the Earth's modern biomass by

Box 1. Energy, Electrons, and Carbon

In microbial physiology, organisms are designated with respect to their core carbon and energy metabolism. There are three components: energy, electrons, and carbon.

Energy: There are two basic ways to harness environmentally available energy: photosynthesis and chemical reactions. The former involves converting light energy into ion gradients for chemiosmotic ATP synthesis (phototrophy). The latter involves chemical reactions to generate ion gradients or to perform substrate-level phosphorylation (SLP) (chemotrophy). Chemotrophs typically utilize redox reactions involving environmentally available electron donors and acceptors. There are exceptions though, for example organisms such as *Propionigenium modestum*, which generates a Na^+ motive force without a redox reaction while decarboxylating methylmalonyl-CoA during succinate conversion to propionate and CO_2 [112], or *Oxalobacter formigenes* [113], which imports dicarboxylic acids in exchange for monocarboxylic acids to generate proton motive force for ATP synthesis.

Electrons: Organisms require a source of electrons for energy metabolism (and biosyntheses). Organisms that live from chemical reactions involving inorganic donors and acceptors only are lithotrophs. If an organism conserves energy from a redox reaction involving at least one organic substrate, it is an organotroph. One example of organotrophy is glucose oxidation with O_2. Methanogens that can grow on isopropanol are another example; they oxidize isopropanol to acetone as an electron source for reducing CO_2 to methane. These methanogens are organotrophs, but they are organoautotrophs because they obtain their carbon from CO_2.

Carbon: Organisms that obtain more than 50% of their carbon from CO_2 alone are autotrophs. Organisms that obtain less than 50% of their cellular carbon from CO_2 are termed heterotrophs. The 50% threshold goes back to a long discussion among microbiologists 30 years ago. Many bacteria synthesize cell constituents from CO_2 and acetate, they obtain about 33% of their cell carbon from CO_2, which might be a maximum for heterotrophs. There are also many carboxylation reactions in the biosyntheses of typical heterotrophs. For example, in both the purine and the pyrimidine ring, one carbon each comes from CO_2, even in the *de novo* synthesis pathways of humans. When autotrophs have vitamin or amino acid auxotrophies, the 50% rule is very helpful.

weight [36], can be excluded as a likely first substrate for heterotrophs because they are chemically too heterogeneous. For example, the Murchison meteorite contains about 2% by weight organic matter, most of which is insoluble macromolecular material (polyaromatic hydrocarbons, which are nonfermentable) accompanied by 23 different classes of organic compounds, each class present at only ppm (parts per million) levels, the most common class being monocarboxylic acids, present in various chain lengths and as various isomers [36]. A common theme of meteorite organics is 'complete structural diversity', meaning that a plethora of isomers for a given chemical formula are present [36], with meteoritic carboxylic acids outweighing the corresponding amino acids by about 50-fold. One can argue that low local concentrations of these compounds could have been overcome somehow, perhaps by circulation through hydrothermal vents and sequestration there. Indeed the volume of today's oceans is circulated through hydrothermal vents about once every 100 000 years [37]. But even if organics from space were concentrated, autotrophs would have had to evolve a vast array of importers, activating enzymes, and catabolic enzymes for each of the individual components in meteoritic organics, none of which would have been present in sufficiently large amounts to support growth. Worse, the vast majority of the organics in meteorites are nonfermentable compounds [36]. Microbial cells, on the other hand, represent a rich, chemically homogeneous and biologically accessible diet of readily fermentable substrates.

What are cells made of? *E. coli* consists of about 55% protein, 25% nucleic acids, 9% lipids, 6% cell wall, 2.5% glycogen and 3% metabolites [38–40] (Table 1). In the absence of evidence to the contrary, Occam's razor would ask us to posit that the first cells had a roughly similar composition. Protein is particularly attractive as the first substrate for the emergence of heterotrophs, not just because it is, weightwise, the main component of cell mass, but because, in autotrophs, the enzymatic machinery for handling amino acids was already in place. As autotrophs, the first cells had complete biosynthetic pathways for all amino acids, bases, and other cell components, such that few evolutionary (enzymatic) inventions, if any, were required to extract energy from those reduced carbon compounds, given suitable environmental conditions. How do these fermentations work?

Amino Acid Fermentations: Bacteria

In bacteria, fermentations of the 20 proteinogenic amino acids involve their degradation to ammonia, CO_2, acetate, short-chain fatty acids, aromatic acids, and small amounts of H_2 [41].

Table 1. Composition of *Escherichia coli* Cells by Dry Weight (g/100 g cells)

Cell Constituent	Stouthamer [38][a]	Lengeler *et al*. [39]	Neihardt *et al*. [40]
Protein	52.4	50–60	55.0
Polysaccharide	16.6[b]		
Glycogen		2.5–25	2.5
Lipopolysaccharide		3–4	3.4
Murein		3–10	2.5
RNA	15.7	10–20	20.5
DNA	3.2	3	3.1
Lipid	9.4	10	9.1
Metabolites, ions		4	3.9

[a]These values are not strictly for *E. coli* as they are taken from a 1968 book by Harold Morowitz, who provided the values in the context of estimating the chemical composition of 'biomass' for thermodynamic purposes, but without explicitly stating to which organism the values apply (probably an average of several).
[b]Here, the polysaccharide value includes 10.3% hexose, which is consistent with the wide range for glycogen reported by Lengeler *et al.* [39].

Energy is conserved via substrate-level phosphorylation (SLP) and via phosphorylation driven by an electrochemical Na^+ gradient (ion-gradient phosphorylation). In the Stickland reaction, carried out by *Clostridium sporogenes* [42], amino acids are fermented pairwise, one is oxidized and the other is reduced [43]. Alanine, valine, and isoleucine reduce glycine to acetate and ammonia or proline to 5-aminovalerate. The latter is fermented by *Clostridium viride* to ammonia, acetate, propionate, and valerate. Alanine, valine and isoleucine are first transaminated with 2-oxoglutarate to glutamate and the corresponding 2-oxoacid, which is further oxidized with ferredoxin to CO_2 and acetyl-CoA, isobutyryl-CoA, or 2-methylbutyryl-CoA, respectively, with SLP via the thioesters yielding the free acids and ATP [44]. The reduced ferredoxin reduces NAD^+ to NADH, mediated by a membrane-bound ferredoxin-NAD reductase – also called Rnf – which generates an electrochemical Na^+ gradient [45,46]. A second NADH is formed by oxidation of glutamate back to 2-oxoglutarate. Both NADH molecules are used to reduce the Stickland acceptors glycine and proline in reactions catalyzed by selenoenzymes.

In many fermentations, the same amino acid serves as electron donor and acceptor. Thus *Clostridium propionicum* ferments 3 alanines to 3 ammonia, 1 acetate, and 2 propionate, whereas 3 serines yield 3 ammonia, 2 acetate, and 1 propionate [47]. This is due to transamination of alanine with 2-oxoglutarate to pyruvate and glutamate, as mentioned above. By contrast, elimination of ammonia from serine directly leads to pyruvate. Whereas the oxidation of pyruvate proceeds as above, its reduction yields D-lactate, which, as lactyl-CoA (2-hydroxypropionyl-CoA), is dehydrated to acrylyl-CoA and reduced with NADH to propionyl-CoA. Such an unusual dehydration has been observed with twelve proteinogenic amino acids: Ala, Ser, Cys, Thr, Met, Glu, Leu, Phe, Tyr, and Trp as well as Gln and His after conversion to Glu [48]. The dehydratases contain [4Fe–4S] clusters and have to be activated by reduced ferredoxin and ATP, mediated by an extremely oxygen-sensitive activator. The formed enoyl-CoAs are reduced with NADH to saturated acyl-CoAs. With exception of acrylyl-CoA, the reduction of an enoyl-CoA is coupled to the reduction of ferredoxin by NADH, a process called electron bifurcation [44]. Even fermentations via the Stickland reaction now appear to involve both electron bifurcation and chemiosmotic energy conservation via the acyl-CoA dehydrogenase/electron-transferring flavoprotein complex and the Rnf complex, as suggested by the genome sequence of *Clostridium sticklandii* [49].

Of the remaining amino acids, Asp and Asn are deaminated to fumarate, which disproportionates: the oxidation proceeds via oxaloacetate and pyruvate to acetate + 2 CO_2, and the reduction proceeds via succinate and 4-hydroxybutyrate to butyrate. Arg hydrolyses to citrulline, which reacts with phosphate to give ornithine and carbamoylphosphate that phosphorylates ADP to ATP via SLP. Lys and ornithine are degraded by special pathways, in which amino groups undergo chemically difficult 1,2 shifts mediated by B_{12}-dependent or *S*-adenosylmethionine (SAM)-dependent radical enzymes [50]. Many of the enzymatic transformations involved in the bacterial amino acid fermentations involve carbon backbone rearrangements and radical intermediates (5′-deoxyadenosyl and ketyl radicals). These reactions are extremely oxygen sensitive and likely represent very ancient biochemical conversions.

Glutamate takes a special place among the amino acid fermentations because it is degraded by two entirely different pathways to identical products [44,51,52]:

$$5\text{Glutamate}^- + 6H_2O + 2H^+ \rightarrow 5NH_4^+ + 5CO_2 + H_2 + 6\text{Acetate}^- + 2\text{Butyrate}^- \quad [1]$$

with $\Delta G_o' = -314$ kJ/mol H_2 and an energy yield of 4.75 ATP, or 0.95 ATP/mol glutamate [44]. In *Acidaminococcus fermentans* glutamate is oxidized with NAD to ammonia and 2-oxoglutarate, which is reduced again to D-2-hydroxyglutarate. This 2-hydroxyacid is dehydrated as CoA thioester to glutaconyl-CoA as indicated above. The decarboxylation of glutaconyl-CoA is catalyzed by a biotin-containing membrane enzyme that converts the free energy of

decarboxylation into an electrochemical Na^+ gradient [53]. The resulting crotonyl-CoA disproportionates to acetyl-CoA and butyryl-CoA, whereby again ferredoxin is reduced via electron bifurcation. Half of the reduced ferredoxin is used for hydrogen formation and the other half adds to the Na^+ gradient mediated by Rnf.

In *Clostridium tetanomorphum* glutamate is converted via a coenzyme B_{12}-dependent carbon skeleton rearrangement to 3-methylaspartate and is further degraded to pyruvate, acetate, and ammonia. Pyruvate is oxidized with ferredoxin to CO_2 and acetyl-CoA, only 20% of which is used for SLP. The other 80% is reduced to butyrate with NADH, generated from reduced ferredoxin via Rnf. Electron bifurcation at the electron-transferring flavoprotein:butyryl-CoA dehydrogenase complex reduces more ferredoxin, which partially gives rise to hydrogen, causing an acetate: butyrate ratio of 3:1. Fusobacteria use the pathway via 2-hydroxyglutarate for glutamate fermentation [51]. *Fusobacterium varium*, however, changes to the 3-methylaspartate pathway if vitamin B_{12} or even cobalt ions are present in the medium [54].

Flavin-based electron bifurcation [44] and chemiosmotic coupling are important in the context of clostridial amino acid fermentation. Both mechanisms appear to be ancient, being employed in the energy metabolism of cytochrome-lacking acetogens. In addition, the coupling site of some acetogens that lack cytochromes [55] is the same Rnf complex as that used by *C. tetanomorphum* and *A. fermentans*. In other words, critical biochemical components of amino acid fermentation more complex than SLP (chemiosmotic coupling and electron bifurcation) were already in place in the autotrophic ancestors of the first bacterial heterotrophs. Only the homogeneous substrates – accumulated protein-rich cell mass – were lacking.

Amino Acid Fermentations: Archaea

Among the archaea, amino acid fermentation is widespread within the Thermococcales (euryarchaeotes) [56] and within the Thermoproteales (crenarchaeotes) [57]. Archaeal amino acid fermenters very often utilize elemental sulfur (S^0) as a terminal electron acceptor [57–59]. In these facilitated fermentations, S^0 affords the microbe an easy means of maintaining redox balance, but ties growth to environments where S^0 is available (geologically active habitats, for example). The overall scheme for energy conservation from archaeal amino acid fermentation is not fully mapped out, but shares differences and similarities with bacteria. Like the bacterial fermentations, archaeal amino acid fermentations entail chemiosmotic coupling [60]. In cases studied so far, coupling in the archaeal amino acid fermentations is thought to involve a reaction similar to that catalyzed by Rnf (an ion-translocating ferredoxin:$NADP^+$ oxidoreductase) but the protein complex, Mbx, is unrelated to Rnf, being related to ion-pumping FeNi-hydrogenases instead [60], NADPH being reoxidized by an NADPH-dependent sulfur reductase, NSR [60]. As in bacteria, amino acids are oxidatively converted into the corresponding 2-oxoacid, and the 2-oxoacid is oxidatively decarboxylated by any one of a large family of CoA-dependent 2-oxoacid oxidoreductases [61], enzymes that can have a fairly broad substrate specificity for different 2-oxoacids. A portion of the electrons from these oxidations ends up on internally generated protons as H_2, another portion ends up on externally supplied S^0 as H_2S (Figure 1), hence the designation as facilitated fermentation. The archaeal amino acid fermentations studied so far appear to lack the kinds of Stickland-type reactions that are found among bacteria, requiring the involvement of external electron acceptors other than amino acids in the archaeal fermentations. Sulfur dependence, that is, the use of S^0 as an external electron acceptor in facilitated fermentations and/or sulfur respiration, seems to be their alternative strategy, one that is quite widespread in energy metabolism among crenarchaeotes [57].

Another important difference between archaeal and bacterial amino acid fermentations entails energy conservation via SLP. While bacteria typically use the classical phosphotransacetylase and acetate kinase (PTA/ACK) system [62] to harness the energy in the thioester bond of

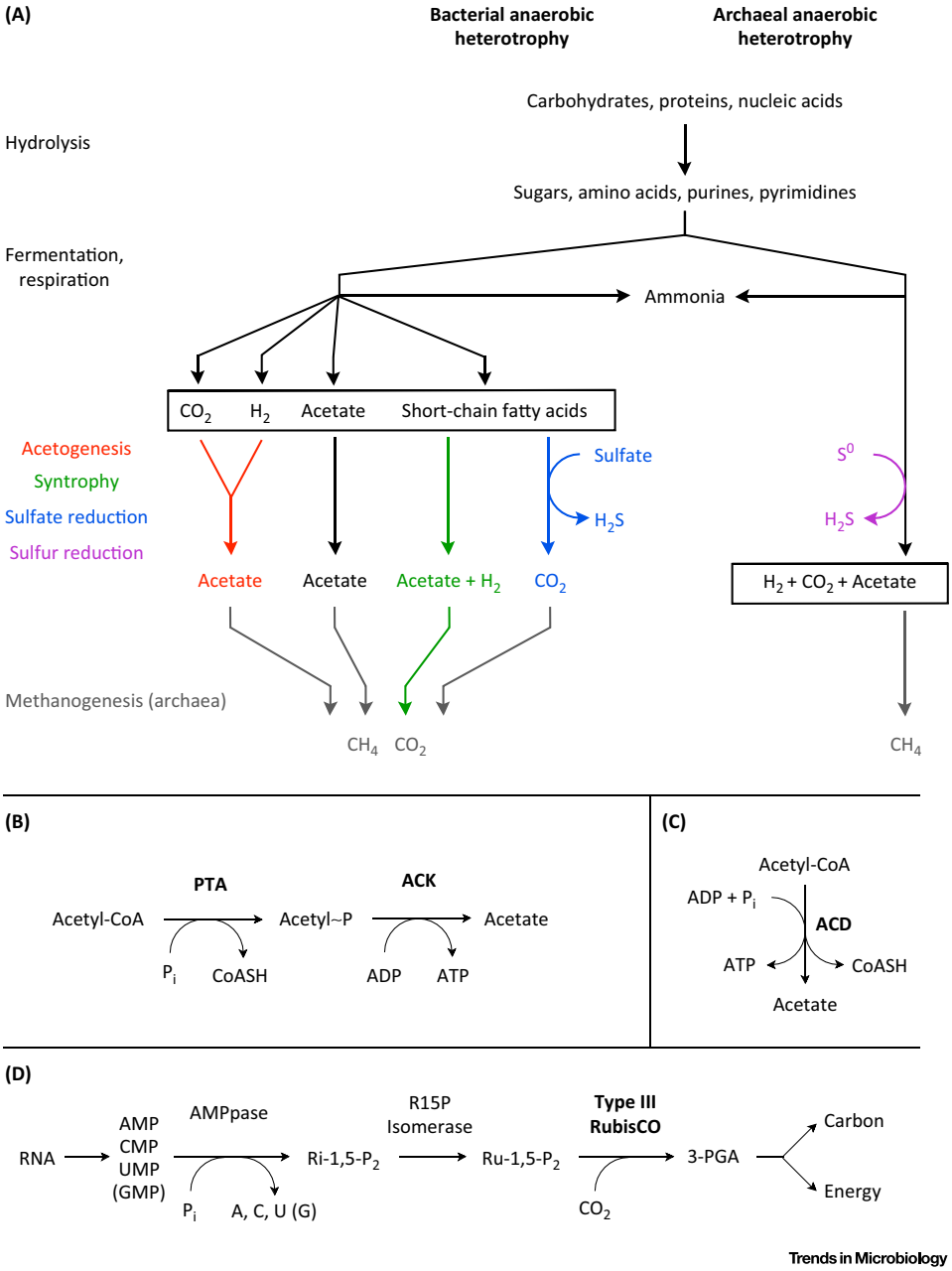

Figure 1. Outline of Bacterial and Archaeal Heterotrophic Pathways. (A) Summarized from [44,57,60,93,96]. For the bacterial fermentations (left) and the archaeal facilitated fermentation (right), the end products are boxed. The subsequent conversion of the fermentation end products via acetogenesis [55,114], syntrophic associations [73], and sulfate reduction [115], as well as the terminal conversion of those products to methane [77], are indicated. Note that some archaea can also use sulfate [57,115], but the use of elemental sulfur has been characterized for the fermentations discussed here [60]. (B) The typical bacterial pathway of ATP formation from acetyl-CoA via substrate-level phosphorylation (SLP) (redrawn from [62]). Abbreviations: PTA, phosphotransacetylase; ACK, acetate kinase; CoASH, coenzyme A (free thiol). (C) The typical archaeal pathway of ATP formation from acetyl-CoA via SLP (redrawn from references [65] and [101]). Abbreviations: ACD, acetyl-CoA synthase (ADP forming). (D) The short pathway of 3-phosphoglycerate formation from the ribose ring in AMP (redrawn from [102]). Abbreviations: AMPpase, AMP phosphorylase; R15P isomerase, ribose-1, 5-bisphosphate isomerase. AMPpase will utilize all four NMPs as substrates, but GMP with lower activity [103,104]. Abbreviations: Ri, ribose; Ru, ribulose; 3-PGA, 3-phosphoglycerate.

acetyl-CoA to phosphorylate ADP (Figure 1B), the archaeal fermenters use the reaction:

$$\text{acyl-CoA} + P_i + \text{ADP} \rightarrow \text{acid} + \text{CoASH} + \text{ATP} \quad [2]$$

which is catalyzed by acetyl-CoA synthase (ADP-forming), or ACD [63] (Figure 1C). The ACD mechanism entails phosphorolysis of the thioester bond to liberate CoA, the free thiol form of CoA (CoASH), generating a noncovalently enzyme-bound acyl phosphate intermediate that phosphorylates ADP, releasing ATP and acetate [64]. ACD is the typical ATP-generating enzyme in the SLP segment of archaeal amino acid fermentations characterized so far. Large families of ACDs with differing substrate specificities occur in *Archaeoglobus fulgidus* [65], *Pyrococcus furiosus* [66], and in *Thermococcus kodakarensis* [67]. The overall fermentative enzyme repertoire just described, including several ACDs, is well represented in the genome of '*Candidatus* Korarchaeum cryptofilum' [68], which grows on amino acids and peptides. ACD rarely occurs among bacterial anaerobes [69], and hyperthermophilic bacteria such as *Thermotoga maritima* use the PTA/ACK system [70], suggesting that ACD is an archetypical archaeal enzyme, not an adaptation to hyperthermophilic lifestyles.

Hydrogen, Heterotrophy, and Shifting Equilibria

The thermodynamic favorability of fermentations depends upon environmental conditions. Under hydrothermal vent conditions (high H_2 partial pressures and moderate temperatures in the range of 50–70 °C), amino acid synthesis from NH_3, CO_2, and H_2 is exergonic [21,71,72]. That is a strong argument in favor of autotrophic origins in the first place because the synthesis of the main building blocks of life had to be thermodynamically favorable. But that leads to an apparent paradox: the first heterotrophs cannot synthesize ATP from amino acid oxidation – and liberate H_2 in the process – under environmental conditions where the reverse reaction (H_2-dependent amino acid synthesis) is exergonic. In the absence of specific syntrophic interactions [73] – a kind of evolutionary specialization – there would have to be sufficient spatial or temporal separation between the site where autotrophs were growing (a high H_2 activity hydrothermal effluent, for example), and a low H_2 activity at the site where amino acid fermentation got started, which would have to be physically or temporally separated from the site of autotrophic growth.

Hydrogen and temperature are probably the keys. H_2 partial pressure can have a decisive influence on the thermodynamics of core bioenergetic reactions among anaerobes, *Thermacetigenium phaeum* [74,75] being a prime example. Under high H_2 it grows as an acetogen, the acetogenic reaction being exergonic under physiological conditions:

$$4\,H_2 + 2\,CO_2 \rightarrow CH_3COOH + 2\,H_2O \quad [3]$$

with $\Delta G_o' = -95$ kJ mol^{-1}. Yet, under low H_2 partial pressure (for example, in syntrophic culture with H_2-consuming methanogens), it grows from the reverse reaction, acetate oxidation:

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \qquad \Delta G_o' = +95\ \text{kJ·mol}^{-1}$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G_o' = -131\ \text{kJ·mol}^{-1}$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad \Delta G_o' = -35\ \text{kJ·mol}^{-1}$$

as written by Hattori *et al.* [74], which works for *T. phaeum* because the acetate-oxidizing reaction is exergonic at low H_2 partial pressures. At H_2 partial pressures of $10^{-4.5}$ atm, the $\Delta G'$ becomes -7.5 kJ mol^{-1}, and becomes even more exergonic at increased temperature, such that at 55 °C the $\Delta G'$ reaches -25 kJ mol^{-1}, sufficient for ATP synthesis [73,74]. Anaerobic acetate oxidation of this kind might have arisen very early in evolution from acetogenic ancestors [14]. The syntrophic requirement for anaerobic acetate oxidation by *T. phaeum* illustrates a point:

Table 2. Values of Gibbs Energy of Reaction, ΔG_r, (J per gram of cells) at 250 bar[a]

Cell Constituent	Amount mg·(g cells)$^{-1}$	ΔG_r (J per gram of cells)				
		25 °C	50 °C	75 °C	100 °C	125 °C
Fatty acids	80	–75	–347	–330	–298	–230
Nucleotides	249	405	214	247	295	380
Saccharides	47	38	–27	–22	–13	8
Amino acids	631	117	–841	–754	–603	–305
Amines	17	14	–15	–13	–10	–3
Total	1024	500	–1016	–873	–628	–150

[a]Summarized from values tabulated by Amend and McCollom [21]. The calculations of Amend and McCollom [21] are based on the mixing of cool, oxidized Hadean seawater (25 °C, pH 6.5, redox midpoint potential, E_h, –300 mV) with hot, reduced alkaline hydrothermal vent effluent (140 °C, pH 9, E_h, –700 mV) in endmember fluids at 250 bar. The values in the column for 25 °C are for Hadean seawater without mixing; the different temperatures result from different mixing ratios [21]. The activities of seawater CO_2 and hydrothermal effluent H_2 underlying their calculations are given in Table 1 of Amend and McCollom [21] as 22.1 and 16 mmol kg^{-1}, respectively. The O_2 levels are assumed to be 10^{-9} mmol kg^{-1} in seawater and 0 in Hadean hydrothermal fluid [21]. Note that oxygen levels are a crucial parameter. Amino acid synthesis and cell mass synthesis, while requiring little if any energy input or even being exergonic under strictly anoxic hydrothermal vent conditions [21], become extremely endergonic even under very mildly oxidizing conditions, such as microoxic conditions corresponding to only 0.001 of present oxygen levels [78].

In the context of the first fermentations, physical or temporal separation of exergonic chemolithoautotrophic growth from exergonic fermentative degradation of cell mass was necessary, for thermodynamic reasons. Hydrogen partial pressure and temperature are probably key variables in that regard.

Importantly, Amend and McCollom [21] examined the thermodynamics for the synthesis of cell mass from H_2, CO_2, and NH_3 under hydrothermal vent conditions of exactly the type that we have in mind here: an alkaline hydrothermal vent with H_2-rich effluent interfacing with CO_2-rich ocean water. They found that the synthesis of cell mass, corresponding to the same general composition as we outlined in Table 1, is endergonic at 25 °C but exergonic at 50 °C, and still exergonic, but less so, at higher temperatures. This is summarized in Table 2, the values in which were extracted with kind permission from Amend and McCollom [21]. The reader might think, at first sight, that this conflicts with the observation that the acetate-oxidizing core bioenergetic reaction of *T. phaeum* shifts from being exergonic under standard physiological conditions (25 °C and high H_2 activity) to becoming exergonic enough for ATP synthesis at low H_2 partial pressures and 55 °C [73,74]. Yet recall that the calculation for *T. phaeum* is for the equilibrium between H_2, CO_2, and acetate, which can be harnessed for the synthesis of ATP to drive physiology forward, whereas the calculations of Amend and McCollom [21] are for the synthesis of cell mass as an end product (Table 2). There is a difference. For example, when growing on H_2 and CO_2, *Clostridium thermoaceticum* (*Moorella thermoacetica*) converts about 24 atoms of CO_2 into acetate for every atom of CO_2 that is incorporated into cell mass [76]; for *Methanothermobacter marburgensis* the value is closer to 40 atoms of CO_2 that are converted into methane for every atom of CO_2 that is incorporated into cell mass, as is readily calculated from the growth yield per methane values reported by Thauer *et al.* [77] – healthy reminders that cell mass is just a minor side product of the main exergonic reaction at the core of energy metabolism. Though the calculations of Amend and McCollom [21] were not performed for lower H_2 partial pressure, it is clear that, in the temperature range of 0–50 °C, equilibrium shifts strongly in favor of CO_2 and NH_3 as H_2 partial pressures drop [78], which is why microbes can harness energy from amino acid fermentations in the first place [79].

Thus, autotrophs that conserve energy through the reduction of CO_2 with electrons from H_2 (acetogens and methanogens) are an energetically favorable starting point of physiological

evolution in microbes in high-hydrogen environments, which favors the synthesis of amino acids [71] and cell mass [21]. At lowered H_2 partial pressure and temperature, the reverse reaction, amino acid fermentations, in the same kinds of anaerobes becomes energetically favorable [44,79], with energy conserved via both SLP and chemiosmotic coupling. Similarly, in modern environments at temperatures above 70 °C, H_2 and CO_2 formation from acetic acid predominates, whereas at lower temperatures acetic acid formation from H_2 and CO_2 predominates. This is because acetogenesis from 4 H_2 and 2 CO_2 (six substrates and one product) becomes less exergonic when the temperature increases; similarly, methanogenesis from acetate at temperatures above 70 °C proceeds entirely via H_2 and CO_2 as intermediates [77].

The first heterotrophic metabolisms thus could have arisen either in H_2-poor cell sediments accumulating around a site of autotrophic growth, or within a region of a vent in which effluent flux, hence geochemical H_2 supply, had come to a halt [80]. The transition from autotrophy to heterotrophy via amino acid fermentation requires very few inventions, as long as the substrate is chemically homogeneous, thermodynamically fermentable, and already on the map of autotrophic metabolism. The examples of *T. phaeum* [74,75] and its predecessor, 'reversibacter' [81], demonstrate the principle of reversed energetics for a given reaction as a function of H_2 partial pressure. The involvement of chemiosmotic coupling and flavin-based electron bifurcation in anaerobic heterotrophic metabolism simply reflects the antiquity of both processes, which are essential for carbon and energy metabolism in anaerobic chemolithoautotrophs [44,55]. In this context, the archaeal facilitated fermentations involving environmental S^0 as an acceptor for redox balance are possibly of broader significance. Sulfur is typically available at geologically active sites and its use would surmount the thermodynamically inhibiting effects of H_2 on fermentations. Did sulfur-dependent organoheterotrophy mark an early crenarchaeal departure from chemolithoautotrophic ancestors? It is something to consider.

How Do Purine and Pyrimidine Fermentations Work?

Many bacteria are known that can satisfy their carbon, energy, and nitrogen needs from purines alone under anaerobic conditions [41,82,83]. Only in comparatively few cases are the overall reaction and the exact energy balance known. One example is *Clostridium acidiurici*, the overall energy metabolic reaction of which, while growing on uric acid, is given by:

$$C_5N_4H_4O_3 + 5.5\,H_2O + 4\,H^+ \rightarrow 0.75\,CH_3COO^- + 3.5\,CO_2 + 4\,NH_4^+ + 0.75\,H^+ \quad [4]$$

with $\Delta G_o' = -144$ kJ per mol uric acid and an energy yield of 1.25 mol ATP per mol uric acid [84]. The details of the intermediates involved in anaerobic purine fermentation [84] are not the focus here. Rather, the focus is on how energy is conserved during the fermentation. Two exergonic reactions are harnessed by *C. acidiurici*, the acetate kinase reaction, and the N^{10}-formyltetrahydrofolate synthetase (formyl-H_4F synthetase) reaction [84], which was long known as an energy conserving step [41,85]:

$$\text{formyl-}H_4F + ADP + P_i \rightarrow \text{formate} + H_4F + ATP \quad [5]$$

with $\Delta G_o' = +8.4$ kJ mol^{-1} [79]. The stoichiometry and energy yield of *C. acidiurici* uric acid fermentation involves electron bifurcation [84]. The genome of *C. acidiurici* also reveals the involvement of chemiosmotic energy conservation via Rnf in the energy metabolism of purine fermentation [86].

For pyrimidine degradations, which do exist [41,82,83], we could not find an example where an anaerobe was reported to satisfy its energy needs from pyrimidines alone or where the balanced overall bioenergetic reaction with the energy yield for pyrimidine fermentation is reported. *Zymobacterium* (*Clostridium*) *oroticum* was found to utilize orotate as a main organic nutrient in the presence of yeast extract [41,87]. *C. sporogenes* can use uracil as an electron acceptor, and it has been suggested that uracil might be able to participate as the electron acceptor in

Stickland reactions [88]. In a natural environmental setting, purines and pyrimidines should be present in roughly equal amounts. In principle, it should be possible to use pyrimidines as a sole energy source. For example, biochemistry textbooks attest that cytosine is readily deaminated to uracil and reduced with NADPH to 5,6-dihydrouracil; two subsequent water additions generate ureidopropionate and β-alanine. In *Clostridium propionicum*, β-alanine is converted to the CoA-thioester and deaminated to acrylyl-CoA that disproportionates reductively to propionyl-CoA and oxidatively via lactyl-CoA, lactate, and pyruvate to acetyl-CoA, which provides ATP [89]. Thus, purines and pyrimidines are – weightwise – important constituents of cells. Purine fermentations are well known, while pyrimidine fermentations, though they should be able to support growth, have been less well studied.

Ribose, the First Abundant Sugar

A prokaryote is about 20% by weight RNA (Table 1), and RNA is about 40% by weight ribose, meaning that a cell is roughly 8% pure ribose. This suggests that ribose clearly would have been among the most, if not the most, abundant early sugar substrate for fermentation. Ribose is indeed an excellent, energy-rich substrate for fermentations and it is furthermore central to many pathways in autotrophic metabolism (including nucleoside and RNA synthesis), meaning that the first autotrophs were unquestionably adept at handling ribose, similar to the situation with amino acids.

The degradations of free pentoses, such as xylose and arabinose enantiomers, by *Haloferax* and *Sulfolobus* have been reviewed [90,91]. These pentose-degradation pathways proceed oxidatively to 2-oxoglutarate, a TCA cycle intermediate. In contrast to the bacterial pentose-degradation pathways, it appears that these archaeal pentose-degradation pathways do not involve direct phosphorylation of free pentoses to pentose phosphates. Archaea generally seem to lack a classical pentose phosphate pathway [92,93], which would be the standard entry point of ribose into bacterial catabolism. Instead they employ a ribulose monophosphate pathway [92,93]. In addition, the utilization of free pentoses is not widely distributed among archaea, appearing to be restricted to halophiles and thermoacidophiles so far and lacking in hyperthermophiles. An oxidative pentose phosphate pathway has recently been described in *Haloferax* [94], whereby haloarchaea have acquired most of the genes that underpin their heterotrophic lifestyle from bacteria [95]. The free pentose degradation pathways of archaea might be evolutionarily recent inventions that arose in the wake of plant cell wall and hemicellulose synthesis. The widespread distribution of gluconeogenic pathways in archaea and bacteria [1,93] suggests that they probably played an important role in the recruitment of pathways for pentose and hexose degradation.

Diversity of C6 Metabolism Suggests That It Came Late

Although glycolysis is often called a universally conserved pathway [8], it is not universally conserved by any means, especially when archaea are considered. There are deep differences in sugar and sugar phosphate metabolism between bacteria and archaea [90,93,96]. The diversity of nonphosphorylated and phosphorylated C6 sugar metabolism both within archaea and across the archaea–bacteria divide [90,93,96], entailing long lists of unrelated and independently arisen enzymes, suggests that C6 heterotrophy arose comparatively late in evolution, and that it arose independently in established lineages of bacteria and archaea. Although archaeal cell walls can have appreciable hexose components, for example glycoproteins from the S layers, glycosylated flagellins, methanochrondoitin, uronic acids and the like [97], the rise of C6 heterotrophy might have occurred in response to the environmental availability of the diverse C6 sugars (possibly photosynthesis-derived) that comprise bacterial cell walls and polysaccharide capsules, constituents of which are ultimately channelled into catabolic C6 pathways. Many of the archaeal enzymes of sugar phosphate metabolism dealing with C6 compounds are members of the ribokinase family [93,96,98].

Despite their many differences, archaeal carbohydrate fermentation pathways tend to converge at pyruvate and acetyl-CoA [93,96], such that acetyl-CoA synthase (ADP-forming) – in addition to its role in amino acid fermentations – also here plays a central role in the terminal energy-conserving step [65,99–101]. This conserved configuration, and the broad distribution of ACD among archaea, points to an ancient role for ACD in heterotrophic archaeal energy metabolism.

Surprising Type III RubisCO

A very intriguing aspect of the origin of heterotrophy concerns archaeal type III RubisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase). The enzyme, long a mystery in archaeal genomes, was recently shown to catalyze the last step in a short pathway (Figure 1D), sometimes encoded in a small gene cluster, that converts nucleotides into 3-phosphoglycerate [102–104]. The first enzyme in the pathway cleaves the nucleotide via phosphorolysis to release the base (a fermentable substrate in the case of adenine), yielding ribose-1,5-bisphosphate, which an isomerase converts into ribulose-1,5-bisphosphate (Ru1,5BP). From Ru1,5BP and CO_2, type III RubisCO generates two molecules of 3-phosphoglycerate – a central intermediate of core carbon metabolism, both in the gluconeogenetic [1] and in the catabolic direction towards pyruvate [93,96]. A simple interpretation is that the type III RubisCO pathway is an ancient relic of the first heterotrophic metabolisms in archaea, RubisCO later being transferred to bacteria, where it is less common among obligate heterotrophs, and where it – in time – ultimately became assimilated into dedicated CO_2 fixation in the Calvin cycle. Type III RubisCO also occurs in nonautotrophic bacterial fermenters from anoxic subsurface environments [105], where it appears to perform the same heterotrophic function identified by Sato *et al.* [102]. This pathway of RNA conversion into 3-PGA is widespread among archaea [102–104] and is now called the ribose bisphosphate pathway [104]. It has recently been characterized in considerable detail in *Thermococcus kodakarensis*, including several new enzymes not discussed here [104].

Lipids, also a notable cell constituent in Table 1, are generally regarded as unfermentable (except in syntrophic associations) and hence stable in the absence of external electron acceptors such as sulfate, nitrate, or oxygen [106]; this is also true for archaeal lipids [107]. Thus, lipids probably just accumulated in early environments, possibly even being reused by the first heterotrophs, until electron transport chains with suitable terminal acceptors to accommodate oxidations of aliphatic compounds had appeared.

Since their origin, amino acid and purine fermentations have been a rich source of reduced nitrogen, and both processes release substantial ammonium [51,84]. However, anaerobic autotrophs are often nitrogen-fixing organisms, and the age of nitrogenase, the enzyme that fixes nitrogen, is debated [108,109]. Clearly, for life to get started, reduced nitrogen had to be available, and experiments show that both transition metal catalysts [110] and hydrothermal vent conditions [111] can provide ample ammonium. But if a geochemical supply of reduced nitrogen comes to a halt, the nature of the challenges facing a microbial community changes, similar to the situation regarding H_2 partial pressures. Local conditions, more than global conditions, were probably decisive in nitrogenase evolution.

Concluding Remarks

Anaerobic autotrophs that use the acetyl-CoA pathway – acetogens and methanogens – are a simple, stable, and 'down to Earth' starting point for the further evolution of microbial physiology. Fermentations were possibly the next step in physiological evolution (see Outstanding Questions) because a minimum of biochemical invention was required to harness the rich reserves of carbon and energy that cells harbor. But for thermodynamic reasons, heterotrophy had to evolve at much lower H_2 partial pressures than those required to support autotrophic growth. Amino acids, bases, and ribose were likely the first fermentation substrates. The seemingly

Outstanding Questions

Phenylalanine fermentation in *Archaeoglobus fulgidus* involves a Stickland reaction: phenylalanine is oxidized to phenylacetate and reduced to phenylpropionate. How widespread are Stickland reactions in archaeal fermentations?

Did the origin of sulfur-dependent facilitated fermentations mark the origin of an early major branch in physiological evolution among anaerobic archaea?

The surprising new ribose bisphosphate pathway (RubisCO-dependent ribose degradation) among anaerobic archaeal heterotrophs looks to be an ancient pathway of carbon and energy supply – who invented it and how ancient is it?

How might H_2 availability have structured the energetic landscape of early microbial diversification?

Are there anaerobes that can satisfy their carbon and energy needs from pyrimidines?

simple fermentations of modern anaerobes continue to harbor surprising reactions, unusual enzymes, and unexpected forms of energetic coupling that harken back to the earliest phases of microbial evolution – the age of anaerobes.

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