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Cite this article: Nitschke W, Russell MJ. 2013 Beating the acetyl coenzyme A-pathway to the origin of life. Phil Trans R Soc B 368: 20120258.
http://dx.doi.org/10.1098/rstb.2012.0258

One contribution of 14 to a Discussion Meeting Issue 'Energy transduction and genome function: an evolutionary synthesis'.

Subject Areas:
evolution, biochemistry

Keywords:
bioenergetics, origin of life, carbon fixation, alkaline hydrothermal vent, methanotrophy, Wood–Ljungdahl pathways

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rstb.2012.0258 or via http://rstb.royalsocietypublishing.org.

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Beating the acetyl coenzyme A-pathway to the origin of life

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Attempts to draft plausible scenarios for the origin of life have in the past mainly built upon palaeogeochemical boundary conditions while, as detailed in a companion article in this issue, frequently neglecting to comply with fundamental thermodynamic laws. Even if demands from both palaeogeochemistry and thermodynamics are respected, then a plethora of strongly differing models are still conceivable. Although we have no guarantee that life at its origin necessarily resembled biology in extant organisms, we consider that the only empirical way to deduce how life may have emerged is by taking the stance of assuming continuity of biology from its inception to the present day. Building upon this conviction, we have assessed extant types of energy and carbon metabolism for their appropriateness to conditions probably pertaining in those settings of the Hadean planet that fulfil the thermodynamic requirements for life to come into being. Wood–Ljungdahl (WL) pathways leading to acetyl CoA formation are excellent candidates for such primordial metabolism. Based on a review of our present understanding of the biochemistry and biophysics of acetogenic, methanogenic and methanotrophic pathways and on a phylogenetic analysis of involved enzymes, we propose that a variant of modern methanotrophy is more likely than traditional WL systems to date back to the origin of life. The proposed model furthermore better fits basic thermodynamic demands and palaeogeochemical conditions suggested by recent results from extant alkaline hydrothermal seeps.

1. Introduction

The quest for the earliest type of biomass-generating carbon metabolism is mostly informed by either or both of two distinct but equally concerned disciplines: palaeogeochemistry and biology. The first of these disciplines provides a picture of the nature of conceivable 'raw material' and free energy for building biomass, whereas the latter considers that extant life still carries the imprint of its origins and that it is possible to distil the ancestral principles out of the variety of extant mechanisms. In the past, attempts towards deducing the nature of the ancestral carbon metabolism were frequently torn between apparently opposing exigencies exerted by geochemistry, on the one hand, and by biology, on the other hand. A popular way out of this dilemma of course is to favour the guiding power of one discipline over that of the other, and material to do so never was in short supply because neither one can yet claim to dispose of definitive results.

More recently, inferences towards an ancestral carbon metabolism have increasingly tried to integrate requirements from both geochemistry and biology [1–5]. Moreover, they have started to acknowledge a further necessary condition for metabolic pathways to emerge, imposed by thermodynamics as pointed out by Schrödinger [6] more than half a century ago and by Boltzmann himself more than a half century before that [7], that is, the need for a strong flow of free energy from the environment which can be coupled to the biomass-producing reactions. Only such a flow of free energy, made productive by being funnelled through coupling devices that convert the dissipation of that free energy to the generation

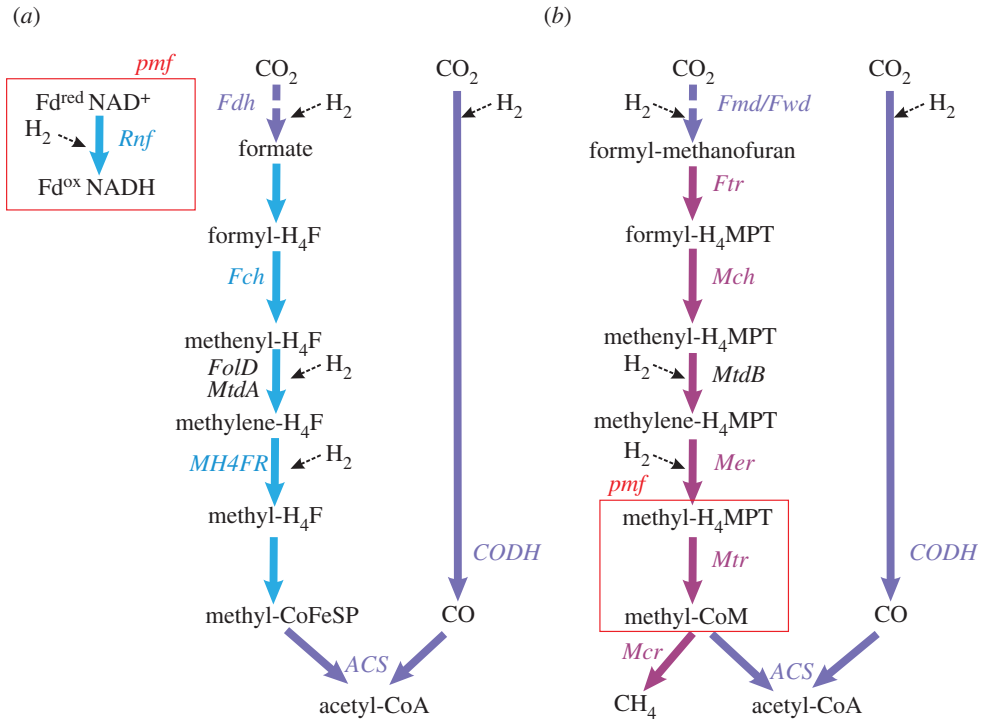

Figure 1. Schematic of the main reaction steps and enzymes involved in acetogenic (*a*) and methanogenic (*b*) WL-type pathways. Steps restricted to Bacteria are marked in bright blue colour, whereas those only found in Archaea are shown in violet. Dark blue stands for reactions and enzymes observed in both prokaryotic domains. Proton motif force (pmf)-generating steps are boxed in red in both reactions.

of 'product' free energy embodied in specific chemical disequilibria [8,9], allows a system to evolve towards the structured, low-entropy state called life while, at the same time, obeying the second law of thermodynamics. To avoid confusion and allow us to focus on the key points of carbon fixation that are the subject of this paper, the bioenergetic aspects of the discussion have been framed in largely conventional terms. This was done notwithstanding the fact that in the companion paper [9] and a preceding paper [8], it was argued at length that these terms are inappropriate for a correct discussion of bioenergetics, the processes conventionally called 'energy conservation' in particular.

In short, thinking about the earliest carbon metabolism and about the origin of life in general has turned to searching extant carbon fixation pathways which do not conflict with geochemical boundary conditions and which, furthermore, directly couple energy metabolism to the biomass-generating process, both of which obviously need to be coupled to the abiotically available sources of free energy.

It appears to us that the most successful takes towards these ends thus far are scenarios building upon the Wood–Ljungdahl pathway (WL) as a blueprint for an ancestral reaction scheme naturally combining carbon- and energy metabolism [1,2,5,9–15]. WL pathways are observed in the extant prokaryotes performing aceto- and methanogenesis, that is, the reduction of CO_2 to acetate and methane, respectively (figure 1). WL pathways are characterized by the fact that, rather than consuming free energy for biomass production, they participate in chemiosmotic potential generation and thus are also free-energy-harvesting processes. In aceto- and methanogens, the WL-type metabolism thus is at the same time a bioenergetic and a carbon-fixing system, whereas in almost all other species, carbon fixation and free energy harvesting occur in distinct pathways and the coupling is ensured by ATP and NAD(P)H. Free energy harvesting in these latter species quasi-exclusively exploits quinone-based electron transfer chains which, through mechanisms of 'free energy conversion' [8] couple the dissipation of the electrochemical disequilibria of various exogenous redox substrates [16] to the production of useful downstream disequilibria (such as the transmembrane proton gradient). The aceto- and methanogenic WL systems considered to be archetypal, by contrast, are devoid of quinone-based membrane-crossing electron transfer.

In addition to its thermodynamic appeal, the WL pathway is at ease with palaeogeochemical boundary conditions because it only requires CO_2 as a carbon source and molecular hydrogen as reductant, both volatiles abundantly present in specific settings on the early Earth [17–20].

As we have tried to convey above, the scenarios stipulating the WL pathway as the earliest energy and carbon metabolism were driven by the aspiration to resolve the tensions between biology, geochemistry and thermodynamics. We feel that this approach is a major step forward from *'ab initio* models' that mostly do not resemble extant life and, more seriously, that, in most cases, lack thermodynamic driving mechanisms without which any origin of life scenario requiring non-equilibrium reactions and/or states is impossible [8].

However, it cannot be denied that a number of tensions persist (and were rightly pointed out as such by opponents of these scenarios) between the presently proposed model building on the WL pathway, on the one hand, and geochemical as well as biological observations, on the other hand. It appears to us that new information and resulting paradigm shifts in both geochemistry and biology during the past decade have only exacerbated these tensions. In the following, we will summarize the most apparent incongruences and then, building upon this inventory, we will try to

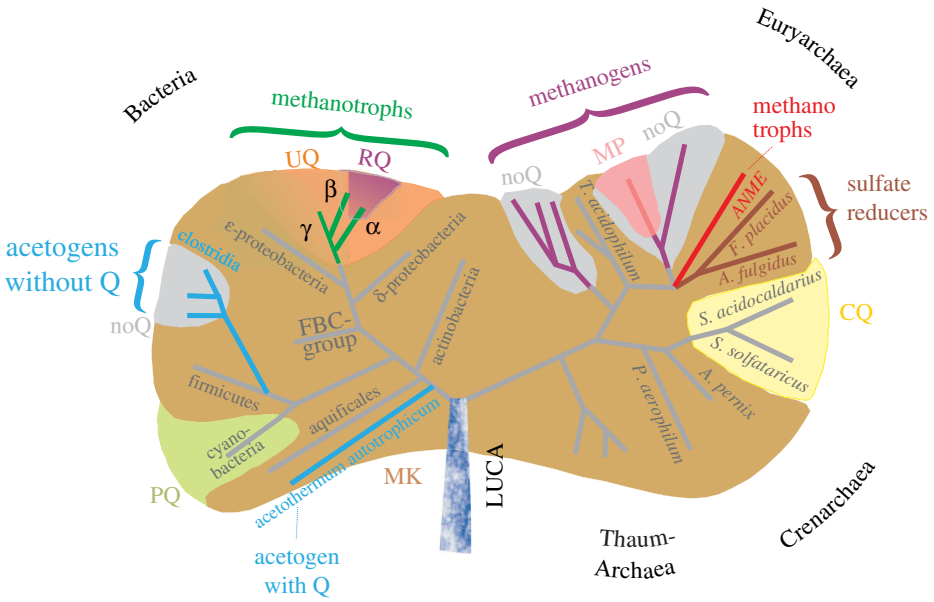

Figure 2. Distribution of free-energy-conserving metabolisms relevant to this article within the prokaryotes. The topology of this schematic phylogenetic tree has been argued previously [16]. Differently coloured regions refer to the presence of different chemical (and electrochemical) types of quinones (or complete absence thereof for the cases of the grey regions). Branches relevant to the topic of this article are coloured similarly to the respective energy metabolisms.

progress towards a model further minimizing clashes between available data from all disciplines.

2. The universal nature of the Wood–Ljungdahl pathway: the touchstone of biology

(a) The molecular make-up of the C_1-body branch in the Wood–Ljungdahl pathway is not conserved between aceto- and methanogens

As pointed out before [3] and as is evident from figure 1, the reactions forming the WL pathway of aceto- and methanogens are deceptively similar and, at first glance, appear to differ mainly by the detailed chemical nature of their C_1-body (formyl, methenyl, methylene and methyl) carriers, tetrahydrofolate (H_4F) in acetogens and tetrahydromethanopterin (H_4MPT) in methanogens (for chemical structures of these carriers, see inset of figure 3). However, acetogenesis appears to be restricted to the bacterial domain, whereas methanogenesis is exclusively found in Archaea (figure 2). This has led to the proposal that it may have been the very diversification of an ancestral hybrid WL pathway into versions eventually yielding acetate or methane as end products (figure 1) that drove the divergence of the last universal common ancestor (LUCA) of all prokaryotes into a bacterial and an archaeal domain [3,21].

Of course, if the aceto- and methanogenic pathways arose from a common ancestor operating at the origin of life, one might expect them to be much more similar to each other than archaeal and bacterial counterparts of other energy and/or carbon metabolisms would resemble each other. This clearly is not the case. In an impressive tour de force by Rolf Thauer's and Ulrich Ermler's groups, almost all enzymes of the C_1-branch in the methanogenic version of the WL pathway (figure 1*b*) have been characterized at atomic resolution of their crystal structures during the past decade [22–29]. As already noted in Martin & Russell [3], searches of the genomes of acetogens for enzymes clearly homologous to those of the methanogenic C_1-branch came up empty-handed with one notable exception, i.e. the initial step of CO_2 reduction which is, in both cases, catalysed by a molybdo/tungstopterin enzyme from the complex iron–sulfur molybdoenzyme (CISM) superfamily [30,31]. However, even these latter enzymes differ substantially with respect to subunit and cofactor composition. Apart from this reaction, all other subsequent reduction steps of C_1-bodies seem to be catalysed by unrelated enzymes in aceto- and methanogens as indicated in figure 1 by blue and violet arrows and enzymes for aceto- and methanogenesis, respectively. This finding makes even clearer the above-mentioned fact that the C_1-bodies methenyl, methylene and methyl are carried by dissimilar molecules, methanopterins in methanogens and folates in acetogens [32].

(b) The carbon monoxide branch of the Wood–Ljungdahl pathway

As shown in figure 1, in WL-type carbon metabolism, only one carbon atom required to form the C_2-moiety acetyl-CoA proceeds through the folate/methanopterin pathways. Although also deriving from CO_2, the second carbon follows an entirely different route. CO_2 is reduced to CO by extremely low potential Ni- and Fe-containing metal clusters in the enzyme CO dehydrogenase (CODH) [33]. This CO is subsequently channelled towards the catalytic site of acetyl-CoA synthase (ACS) where it is condensed with the methyl group produced via the C_1-body pathway to yield acetyl-CoA [33]. The strongly reducing equivalents necessary for CO_2 reduction to CO are produced through electron bifurcation [34]. In striking contrast to the C_1-body pathway, the enzymes CODH and ACS are homologous in aceto- and methanogens. Phylogenetic analyses [35,36] furthermore indicate that these enzymes likely were already present in the LUCA and have been inherited mainly vertically into extant WL-pathway species. To emphasize this fact, the corresponding enzymes and reaction arrows are similarly coloured in dark blue for both the aceto- and the methanogenic pathways in figure 1. We are thus left

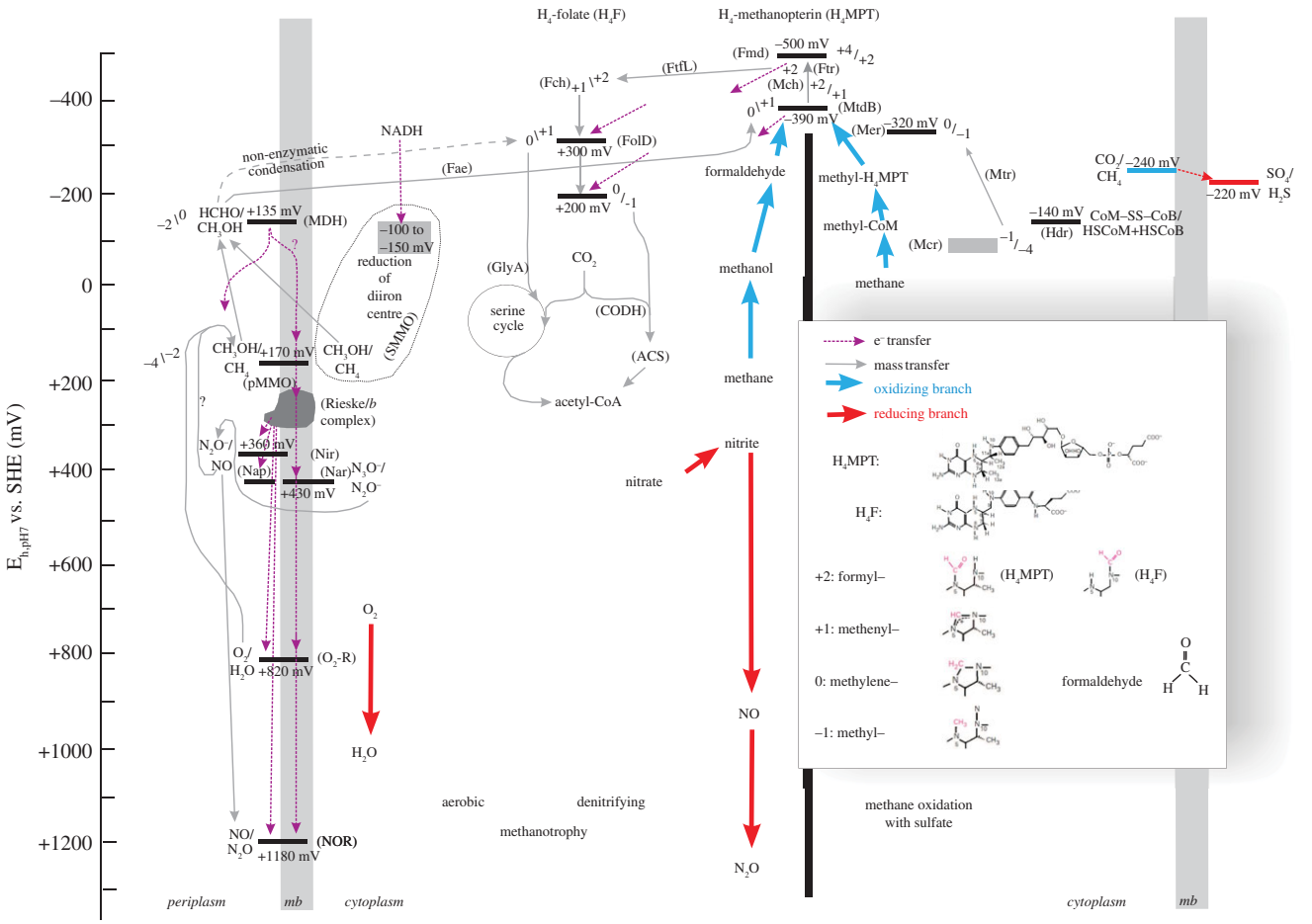

Figure 3. Reaction scheme, represented on an electrochemical scale, of the methanotrophic pathways studied in Bacteria (left-hand side scheme) and in Archaea (right-hand side scheme). The positions of the terminal carbon-fixating processes via the serine-cycle or via CODH/ASC are not meant to reflect electrochemical properties but are positioned for ease of schematic representation. Enzyme abbreviations: MDH, methanol dehydrogenase; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; Nir, nitrite reductase; Nar, membrane-bound nitrate reductase; Nap, periplasmic nitrate reductase; O_2-R, oxygen reductase; NOR, nitric oxide reductase; Fae, formaldehyde-activating enzyme; Mcr, methyl-coenzyme M reductase; Mtr, coenzyme M methyltransferase; Mer, methylene-H_4MPT reductase; Mch, methenyl-H_4MPT cyclohydrolase; Ftr, formylmethanofuran:H_4MPT formyltransferase; FtfL, formyltetrahydrofolate ligase; Fch, methenyl-H_4F cyclohydrolase; FolD, 5,10-methylene-tetrahydrofolate dehydrogenase; GlyA, serine hydroxymethyltransferase; CODH, carbon monoxide dehydrogenase; ACS, acetyl-CoA synthase; Hdr, heterodisulfide reductase. The vertical light grey bar represents the cytoplasmic membrane. Peri- and cytoplasmic spaces are as indicated in the bottom of the figure.

with the puzzling observation that only half of the WL mechanism apparently has the credentials for a mechanism potentially reaching back to the origin of life, whereas the other half clearly does not.

(c) The free-energy-converting steps in aceto- and methanogens are dissimilar

All life uses the chemiosmotic principle of ATP formation driven by a proton- (or sodium-) motive-force across a lipid membrane [5,16,37]. The energy-coupling step in the WL pathway of methanogens (lacking methanophenazine, see §2(d)) was described by Thauer and co-workers [38] about 10 years ago, whereas the corresponding process in acetogens was elucidated only recently [39,40]. These enzymatic steps, mediated by methyltransferase (Mtr) in methanogens and by the Rnf-complex in acetogens (the name Rnf historically derives from 'Rhodobacter nitrogen fixation'), are indicated by red boxes in figure 1. As is obvious from figure 1, these steps are completely unrelated. Because the free energy content of the substrate couples exploited by aceto- and methanogens is just sufficient for a single charge-translocating step [16], these enzymes are probably the only chemiosmotically coupling entities in both systems, a finding that emphasizes just how crucial their respective roles are in free energy conversion. The dissimilarity of this pivotal reaction step in aceto- and methanogens is thus a further entry into the list of observations suggesting a deep cleft between the two types of WL pathways.

The recent finding that a Rnf complex plays a crucial role in a methanogenic Archaeon [41] might be taken to contradict this line of argument. A search of protein sequence databases was performed using BLAST to identify the occurrence of functional equivalents (homologues) of *rnf* genes among organisms. This search detected homologues only in methanosarcinales, i.e. the group of methanogens that contain the liposoluble hydrogen carrier methanophenazine (figure 2). Closest BLAST hits were the *rnf* genes from clostridia and, intriguingly (see below), γ-proteobacterial methanotrophs. As mentioned in Schlegel *et al.* [41], this finding suggests the likelihood of lateral gene transfer rather than vertical inheritance. Considering the crucial role of the Rnf complex in free energy conversion at the thermodynamic limit, the evolutionary history of this complex certainly requires a dedicated and in-depth study. Because no methanophenazine-free methanogen containing the Rnf complex has yet been found, we maintain our argument that the free-energy-conserving

steps in WL-type aceto- and methanogenesis appear to be distinctly dissimilar.

(d) The phylogenetic distribution of free-energy-harvesting systems make Wood–Ljungdahl pathways look derived rather than ancient

As discussed elsewhere [16], a number of observations indicate that the LUCA may already have harboured quinone-mediated electron transfer chains. Almost all species of prokaryotes contain quinone-based chemiosmotic chains (figure 2) and many enzymes that reduce quinones or oxidize quinols appear to have pre-LUCA origins based on their molecular phylogenies [31,42–45]. Quinones are the quintessential mediators of proton-motive-force generation [16] and it is difficult to conceive a raison d'être for pool quinones in membranes different from the build-up of chemiosmotic potential.

On this basis, there is every reason to believe that the LUCA already had fully-fledged systems for the generation of membrane potential at her disposal. The independent origins of coupling enzymes in aceto- and methanogens then lose their possible rationalization and the fact that acetogens and methanogens use dissimilar ways to generate chemiosmotic potential makes them look much more like derived types of energy metabolism rather than founding systems.

We have illustrated this fact by overlaying the phylogenetic distribution of relevant types of energy-converting systems onto a schematic tree of prokaryotes. It is noteworthy that the detailed topology of this tree certainly is a matter of debate but of no importance to what we seek to convey here. Figure 2 demonstrates that production of chemiosmotic potential via menaquinone-mediated electron transfer is the dominant scheme in prokaryotes. As discussed in Schoepp-Cothenet *et al*. [16], an increasing body of results suggests this mechanism not only as dominant but also as ancestral to the divergence of the Bacteria and the Archaea. Quinone-free methanogens, by contrast, are found in the overwhelming majority of recent phylogenetic trees of the Archaea as higher branching orders [46]. The divergence into Euryarchaeota (containing, among others, methanogens) and Crenarchaeota (entirely devoid of methanogens), visible even on the earliest trees built from a much smaller number of species [47], has made methanogenesis as the ancestral trait of the archaeal domain doubtful. Acetogens, in turn, are only found in Bacteria, and the bulk of the archetypal acetogens considered in WL scenarios for the origin of life, i.e. those devoid of quinone-based electron transfer chains, are mainly clustered in the clostridia that generally are not considered to be a very early branching phylum [48]. Admittedly, a representative of one of the earliest branching phyla, the obsidian pool-1 (OP-1) group, has recently indeed been shown to contain all traits of the acetogenic lifestyle [35], but this organism is an acetogen with quinones and its genome contains enzymes involved in electron transfer to high-potential acceptors. Furthermore, the phylogenetic affiliation of several enzymes taking part in these quinone-mediated electron transfer chains such as the Rieske/cyt*b* complex (F. Baymann 2012, personal communication) or the Nar-type nitrate reductase (B. Schoepp-Cothenet 2012, personal communication) indicates that these enzymes branch very early in their respective phylogenies in line with vertical inheritance and thus pre-LUCA presence of these enzymes. The OP-1 species *Candidatus Acetothermum autotrophicum* therefore corroborates the scenario of the ancestral character of quinone-based electron transport and re-emphasizes the apparent late emergence of quinone-free acetogens. The global picture suggested by figure 2 therefore is that of WL-type acetogenic and methanogenic pathways as derived from a quinone-based electron transfer chain.

3. New results from geochemistry widen the range of redox- (and carbon-) substrates for early life

(a) Previously neglected reducing substrates

A major appeal of the WL pathways as encountered in aceto- and methanogens is their reliance on the sole substrates H_2 and CO_2, the presence of which in hydrothermal settings on the Earth, now and then, is beyond reasonable doubt. However, the full inventory of redox substrates available at life's inception may have been richer than previously assumed. The discovery and further expeditions to actually existing submarine alkaline hydrothermal systems [49,50], corresponding to alkaline seeps hypothesized to exist both in the Hadean and the present day [51,52], provided us with a wealth of information as to which elements and molecules are delivered to the observed hydrothermal porous, chimney-like structures from below via the serpentinization process. The data obtained from the Lost City field, in fact, turned out to loosen the energetic and chemical constraints previously thought to prevail in alkaline hydrothermal systems. Among the plethora of chemical components found were high concentrations of methane [53,54].

From chemistry, it was long known that methane was produced at relatively high temperatures from H_2 and CO_2, catalysed by Fe- and Ni-rich minerals [55–58]. This fact was indeed one of the crucial foundations of the 'aceto-and methanogenesis-early' scenario which stipulated that methanogenesis merely was an optimization and quickening of the methane-generating geochemical processes [3]. The actually observed concentrations of up to 2 mmol l^{-1} of methane at Lost City are intriguing. However, although molecular hydrogen (E_m of $H_2/H^+ = -413$ mV) is thermodynamically capable of reducing CO_2 (e.g. to acetate with $E_m = -290$ mV or to methane with $E_m = -240$ mV), CH_4 cannot serve as reductant for CO_2 (because it is not reducing enough) or as oxidant for H_2 (because it already is maximally reduced) and therefore cannot enter the stage of energy metabolism unless a more positive potential electron acceptor is available.

(b) Likelihood of oxidants on the early Earth

Methane is an electron-donating substrate in extant-energy-producing metabolisms and is even used to provide carbon for biomass as will be detailed in §6. The presence of sufficiently oxidizing substances, however, is required for methane to be able to act as a redox substrate. What might they have been? Of course, such oxidized electron acceptors are abundant on our present-day planet owing to the generally high redox state of the biosphere entailed by the rise in O_2 2.5 billion years ago. But not so in the Hadean. Therefore, information gathered at Lost City with respect to the amounts of oxidants will not help—their nature and amounts inevitably not reflecting those on the pristine Earth.

We are therefore obliged to rely on inferences informed by the chemical and geochemical sciences when trying to assess whether and, if so, which, oxidants may have been present on the early Earth. And the first point to note is that if oxidants were present in the setting of the alkaline hydrothermal mounds, they must have been delivered from the outside ocean, because the strongly reducing state imparted by the serpentinization reaction excluded the possibility of oxidants in the hydrothermal fluid. But there is good reason to believe, as we summarize next, that reasonably strong and abundant oxidants, such as nitrogen oxides and nitrogen oxyanions, may have been available in the ocean of the Hadean.

Palaeogeochemists have in the past come up with a series of mechanisms for the large-scale production of nitric oxide from atmospheric N_2 and CO_2 ranging from volcanism through bolide impacts to lightning [20,59]. The energy necessary to activate the relatively inert gases N_2 and CO_2 in all these cases comes from either heat or electrical discharge, or both. The amounts proposed to have been produced via these processes are indeed of global scale and we have previously calculated, based on the estimates put forward for these reactions, that ocean concentrations of nitrate and nitrite, the most likely stable aqueous products of atmospheric NO equilibrating with the pristine ocean, could have attained a few hundred micromoles per litre [44].

It seems noteworthy that the availability and use of nitrate and nitrite as oxidants during the days of the LUCA gain some support from extant biology. Molecular phylogenies of several enzymes involved in the modern denitrification pathway, i.e. anaerobic respiration of nitrate and nitrite, show signs of pre-LUCA origins (reviewed in van Lis *et al.* [45]). Other possible electron acceptors available in the Hadean Ocean were photo-oxidized iron and manganese oxide clusters [60,61] as well as elemental sulfur and sulfate [62].

However, although S_0 seems a very possible candidate, basic energetic/thermodynamic considerations [16] and the apparent difficulties of extant life in using S_0 and even the more oxidizing sulfate ion for methanotrophy [63,64] make us reluctant to consider sulfur compounds as essential oxidants for early life.

In summary, a strong argument can be made from geochemistry that oxidants with standard redox midpoint potentials of more than 0 mV were likely delivered through the ambient ocean to chemical reactions occurring at the locations where alkaline hydrothermal fluids met ocean waters. Oxidation of methane and ammonia, another volatile likely present in alkaline hydrothermal fluids [65], resembling extant bioenergetic pathways, is therefore a possibility which needs to be taken seriously when inferring the ancestral types of metabolism.

4. Autotrophic carbon dioxide fixation: the conundrum of pathway multiplicity

Autotrophic life is defined as deriving carbon atoms for biomass exclusively from CO_2. Carbon dioxide is indeed practically ubiquitous in all habitats on our planet and certainly was even more so on the early Earth given that the atmospheric pressures may have been as high as 10 bar [66,67]. Owing to the very low redox potentials of the reduction steps converting carbon in CO_2 (with a formal oxidation number of +4) to biomass-available carbon (where carbon mainly is 0 to −3), autotrophic CO_2-fixation is a bioenergetically challenging reaction; hence, life's avidity to use carbon pre-reduced by different ('heteros' in ancient Greek) organisms to the so-called organic molecules, a lifestyle consequently termed heterotrophy. Organic soup scenarios stipulate that sufficient quantities of organic molecules may have been produced in Miller–Urey-type reactions to allow heterotrophy as the ancestral system of biomass production. Apart from all the controversy concerning the soundness of the starting conditions for Miller–Urey experiments, it has in the recent past been argued that organic soup scenarios for the origin of life are severely at odds with the second law of thermodynamics [3,68] and these models are therefore not considered here. More recent approaches to life's emergence [1–3,11,15] have consequently concentrated on autotrophic carbon fixation and assumed that one or more of the known extant autotrophic pathways can serve as at least a partial model of how it was first achieved. However, at least six distinct autotrophic carbon fixation pathways have been elucidated during the past few decades [15,69,70].

This multiplicity of pathways inevitably raised the question as to which of these possibly functioned in emerging life. As detailed at the beginning of this article, the WL pathway found in the acetogens and the methanogens is presently favoured owing to its simplicity, far-going reliance on inorganic cofactors and chemiosmotic potential-generating second nature. If this is how life began fixing carbon, we are led to wonder why an ancestral WL pathway has not become life's one and only principle for biomass production. An argument that might be put forward by proponents of the WL scenario is that this pathway requires a relatively strong reductant which in the archetypal versions of methano- and acetogenesis is molecular hydrogen. However, both methano- and acetogens using less-reducing substrates are found in Nature. Furthermore, all living cells, including those not having access to very negative redox substrates, need to generate strongly reducing metabolites (e.g. NAD(P)H, $F_{420}H_2$) for a plethora of crucial cellular processes and are capable of doing so through reverse electron transfer and/or electron bifurcation. The presence of H_2 thus obviously is not an absolute prerogative to the WL pathway to function. Another objection may be that several key enzymes in the WL pathway are extremely oxygen-sensitive and that the advent of O_2 in the biosphere obliged life to search for other solutions. However, even strict anaerobes have dissimilar autotrophic CO_2-fixation mechanisms (see, e.g. the reverse tricarboxylic acid cycle in the obligatorily anaerobic green sulfur bacteria [71]).

The multiplicity of autotrophic CO_2-fixation pathways therefore indeed represents a major puzzle in current scenarios stipulating an autotrophic origin of life. Braakman & Smith [72] have recently proposed that the ancestral CO_2-fixation pathway might have been a combination of parts of the extant autotrophic systems and that this ancestral multivalent metabolism was subsequently streamlined by numerous selective losses to yield the extant multiplicity of (in this model) seemingly different principles. We have to admit that this scenario is attractive in providing a simple and comprehensive answer to the question of why there are so many different pathways. However, provocative as this may appear, an even simpler rationalization to the multiple pathways conundrum does exist: none of the traditional autotrophic CO_2-fixation pathways actually operated in the LUCA!

Guided by the geochemical compositions determined in hydrothermal fluids, we propose to reconsider the traditional definition of autotrophic versus heterotrophic carbon fixation. Methane with its strongly reduced carbon (formal oxidation state of −4) appears to be produced in the lithosphere and delivered to hydrothermal systems through entirely abiotic reactions [57]. However, in the framework of the current definition, methane is a C_1 organic molecule. Although succinate, pyruvate or acetate clearly fall into the organic realm (if this is taken to mean 'produced by and found in organisms'), such a classification is much less straightforward for the shorter chain representatives formate and, most obviously, for methane. We would hold that the problem of this somewhat arbitrary line between inorganic and organic carbon constitutes one (psychological) obstacle, among other more physical ones as discussed in §5, to considering methane oxidation as a source for both energy and carbon to earliest life.

In the following, we will try to assay extant types of methane metabolism for their possible relevance to origin of life scenarios. Hydrothermal systems on our present-day planet show H_2/CH_4 ratios which in general make CH_4 formation from CO_2 and H_2 thermodynamically favourable [73]. This is in line with the above-mentioned WL scenarios stipulating that early life simply quickened this reaction. However, if indeed oxidants were present, reliance merely upon the traditional WL pathway would make nascent life miss out on a major potential source of free energy. But, it seems unlikely to us that it would have done so. From its very first stirrings, we argue, pre-life was as much, and as essentially, a dynamic industry of far-from-equilibrium processes and structures as it has been ever since; and thus nascent life was no less dependent on abundant sources of chemical free energy to drive and maintain its essential endergonic reactions and non-equilibrium states than is all extant life [8]. If alkaline hydrothermal vents were indeed, as described in §3(a), richer in energy substrates than the mere CO_2 and H_2 couple, WL-pathway-based life would have done rather poorly in terms of exploiting the available disequilibria. By contrast, methanotrophic organisms seem much better suited for using such environments. The vast majority of present-day methanotrophs, however, use molecular oxygen to oxidize methane. This very fact probably represents the second major obstacle for counting methanotrophy in as a putative primordial energy metabolism. However, the discovery of anaerobic methane-oxidizers both in the archaeal [74–78] and, more recently, in the bacterial domain [79,80] has now done away with this obstacle.

Let us therefore examine more closely whether these anaerobic methanotrophs might provide promising models for primordial metabolism in an alkaline hydrothermal mound.

The archaeal representatives, the so-called ANME groups (stands for anaerobic methane oxidizers), are found to be obligate symbionts with sulfate-reducing bacteria (SRB). It is in fact Bacteria that carry out the reduction of the terminal oxidant, sulfate [66]. The ANMEs themselves seem to reverse the methanogenic pathway and contain, just as methanogens do, the CoB–CoM heterodisulfide as an internal terminal redox compound [77,81,82] (figure 3, right-hand chain of reactions). Interestingly, disulfide (HS_2^-) was recently suggested to be the redox-vector-linking methane oxidation in the ANME-2 subgroup to sulfate-reducing δ-proteobacteria [82]. Although this suggests that ANME-2, in principle, might carry out methanotrophy without the syntrophic partner (a fact which we would anyway consider a prerequisite for the emergence of the pathway), its sustainable efficiency seems to be on the thermodynamic edge and the mechanism becomes truly viable only through the association with a phylogenetically completely unrelated class of bacteria pulling the product equilibrium towards the required ratios. We believe that these findings render the methanotrophic system of ANMEs quite unattractive as paradigms for primordial energy metabolism.

5. Denitrifying methanotrophy: an extant energy metabolism 'ready-made' for alkaline hydrothermal vents

In 2010, the isolation of the first anaerobic methane oxidizing member of the Bacteria was reported and its genome was reconstructed from metagenomic data [80]. As judged from its gene content, this organism, named *Methylomirabilis oxyfera*, contains all the key enzymes that are also involved in aerobic methane oxidation [84–92], that is, methane monooxygenase (more specifically its membrane-bound version pMMO), methanol oxidase and the formaldehyde-activating enzyme (Fae). In contrast to ANMEs, where methane is transformed to methyl-CoM and then transferred to H_4MPT for further oxidation, *M. oxyfera* uses methane via two consecutive $2e^-$ oxidations yielding first methanol and then formaldehyde just as aerobic methanotrophs do (figure 3, left-hand side scheme). It is only at the level of formaldehyde that transfer to the C_1-body carrier H_4MPT occurs. Because the redox reaction from CH_4 to CH_3OH occurs at +170 mV, *M. oxyfera* requires 'truly' high-potential acceptors, more oxidizing than the sulfate/H_2S couple ($E_m = -217$ V) used by ANMEs. However, redox potential is only part of the problem. Whereas activation of the strongly inert methane molecule by coenzyme M (CoM) in ANMEs (figure 3, right-hand scheme) is a bi-molecular reaction, the oxidation of methane to methanol requires the participation of an oxygen atom and proceeds in aerobic methanotrophs according to the scheme $CH_4 + O_2 + 2e^- + 2H^+ \Leftrightarrow CH_3OH + H_2O$. Thus, while the carbon atom in methane (−4) is indeed formally oxidized to methanol (−2), yet the overall reaction is a reduction wherein the required electrons serve to activate an O_2 molecule permitting it to react with the methane.

If *M. oxyfera* uses the same enzymatic pathway as its aerobic counterparts, where then does the oxygen atom come from in an anaerobic environment? The key to this problem lies in the type of oxidant used by *M. oxyfera*, the nitrogen oxyanion nitrite. The groundbreaking work by Ettwig *et al*. [80] has indeed shown that the oxygen atom incorporated into methane derives ultimately from nitrite (NO_2^-) through an as yet poorly understood mechanism. Ettwig *et al*. have proposed the existence of a hypothetical enzyme disproportionating two NO molecules (arising from the reduction of nitrite via the denitrification enzyme nitrite reductase) into N_2 and O_2. More recently, Chen & Strous [93] have suggested the possibility that rather than from NO, O_2 and N_2 may be produced from N_2O. We would like to note that the examples of enzymes transforming both O_2 and NO, such as the O_2/NO-reductase superfamily [94] or cd_1-nitrite reductase (cd_1-Nir) [95] suggest the possibility

of NO itself substituting for O_2 in the methane-to-methanol conversion via $CH_4 + 2NO \Leftrightarrow CH_3OH + N_2O$ (as indicated by the arrow next to the question mark in the left-hand side scheme of figure 3). For the case of the O_2/NO-reductase superfamily, it has indeed been proposed, based on phylogenetic evidence, that the NO-converting enzyme pre-dates in evolutionary terms the O_2-reducing one [44].

Whatever the detailed mechanism for using the oxygen atom of nitrite in the oxidation of methane to methanol may finally turn out to be, the overall reaction carried out by *M. oxyfera* provides a biological proof of principle for the functionality of the methanotrophic pathway even under anaerobic conditions, provided that nitrogen oxyanions or nitrogen oxides are available. Although Fe^{3+} or Mn^{4+} may well be electrochemically viable oxidants for methane conversion, the lack of an activatable oxygen atom in such oxidation reactions precludes a pathway through methanol and formaldehyde as shown in figure 3 (left-hand side scheme). We therefore assume that methane oxidation putatively pulled by Fe^{3+} and Mn^{4+} [96] proceeds through the right-hand side scheme pathway of figure 3, that is, possibly in a syntrophic manner as for the ANME/SRB association. As we will show in §7, phylogenetic and phylogenomic evidences favour the methanol/formaldehyde pathway as an ancient, possibly pre-LUCA, mechanism, whereas the ANME-type methanotrophy may be derived from methanogenesis. And methanogenesis itself may ultimately derive from the sulfate-dependent methanotrophic pathway shown in figure 3 (right-hand side scheme).

Given this possible deep ancestry of the methanol/formaldehyde-mediated methanotrophic pathway, let us now review the fate of the formaldehyde carbon in the subsequent reaction steps of this mechanism.

6. The methane carbon atom is integrated into biomass in a process resembling the Wood–Ljungdahl pathway with respect to several features

The formaldehyde molecule is loaded onto tetrahydromethanopterin (H_4MPT) by the so-called Fae to yield methylene-H_4MPT (figure 3, left-hand side scheme). Because carbon remains at the formal oxidation state of 0 during this reaction, no reducing equivalents are liberated in this step. Methylene-H_4MPT is then consecutively oxidized in two single-electron transitions, first to methenyl-H_4MPT (with a formal charge of −1) and then to formyl-H_4MPT (−2), finally yielding formate (via formylmethanofuran). Formate represents a redox branch point with a fraction being fully oxidized to CO_2. This fraction represents the part of methane oxidized to exclusively serve bioenergetic ends. The remaining fraction of the formyl moiety is transferred to H_4F by the enzyme formyl-H_4F-ligase and from there on is re-reduced through the canonical cascade of C_1-bodies on H_4F. In this context, it is noteworthy that formaldehyde also condenses spontaneously and non-enzymatically with H_4F to yield methylene-H_4F as indicated by the dashed arrow in figure 3 [97], thereby short-circuiting the detour through formate. However, in proteobacterial methano- and methylotrophs, this direct reaction is obviously outcompeted by the enzymatic pathway, and the enzymatic acceleration is apparently of sufficient use to the parent species to warrant spending ATP in the detour [92,97]. Incidentally, we note that a great deal of the information reviewed here has been gathered on methylotrophs. Methylotrophs lack the first enzymatic reaction from methane to methanol but are similar to methanotrophs with respect to all subsequent reaction steps.

Methano- and methylotrophs appear to use several different [85] and interconnected [92] pathways for carbon fixation (for an excellent representation of these mechanisms, see Smejkalová *et al.* [92]). For the purpose of this article, the most pertinent of these is the so-called serine-cycle which, in a nutshell, condenses the C_1-body of methylene-H_4F (oxidation state of 0) with a CO_2 molecule and glyoxylate to yield acetyl-CoA (figure 3, left-hand side scheme). In this manner, methane and CO_2 contribute roughly equally to biomass. The fact that biomass in methano- and methylotrophs is made up from both methane- and CO_2-carbon has been known for almost half a century [98] and has been confirmed since [91].

The branch yielding methylene-H_4F in methanotrophs is basically indistinguishable from the C_1-body branch in the WL pathway of acetogens (figure 1). Of course, the common substrate formate is not produced from CO_2 by the molybdopterin enzyme formate dehydrogenase, but instead is the result of the oxidation cascade starting at methylene-H_4MPT, i.e. the reversal of the reactions in methanogenesis based on H_4MPT and on the respective enzymes (as indicated in figure 3).

Just as in the WL pathway, the C_1-body on H_4F is condensed in an asymmetric manner with carbon from CO_2 delivered via a second reaction sequence. The main difference lies in the fact that in methanotrophy carbon enters the serine-cycle at the formal oxidation state of 0 (methylene-H_4F), whereas in the WL pathway of acetogens, a C_1-body at a higher (by one negative charge) reduction state, methyl-H_4F (−1), is fused to CO by ACS. Obviously, this simpler reaction using methyl-H_4F and CO from CO_2 (via CODH) cannot work in the well-studied extant methanotrophs owing to the extreme sensitivity to O_2 of the nickel-containing ACS/CODH enzyme dyad.

Therefore, the oft-mentioned attractive aspect of the WL pathway to be at the same time an energy-converting and a carbon-fixating mechanism is also a feature of methanol/formaldehyde-based methanotrophy.

7. A case for the ancestry of the methanol/formaldehyde-based methane oxidation pathway over methano- and acetogenesis as well as over methanotrophy on sulfate

The C_1-carrier H_4MPT as well as the enzymes redox-transforming these carbon moieties were discovered and characterized in methanogenic Archaea and initially considered to be specific to methanogens [99]. They were, however, subsequently shown to occur in methano- and methylotrophs from the bacterial domain [100], in planctomycetes [101] and in methane-oxidizing Archaea of the ANME group [77].

The essential cofactors of acetogenesis, methanogenesis and methanotrophy therefore are present in both prokaryotic domains. As discussed in §2(a) (see also figure 2), homoacetogenesis so far is found only in Bacteria, whereas methanogenesis appears to be confined to Archaea. Anaerobic methanotrophic pathways, however, are observed in both domains, although passing through differing intermediates in going from the −4 state of carbon oxidation (methane) to the

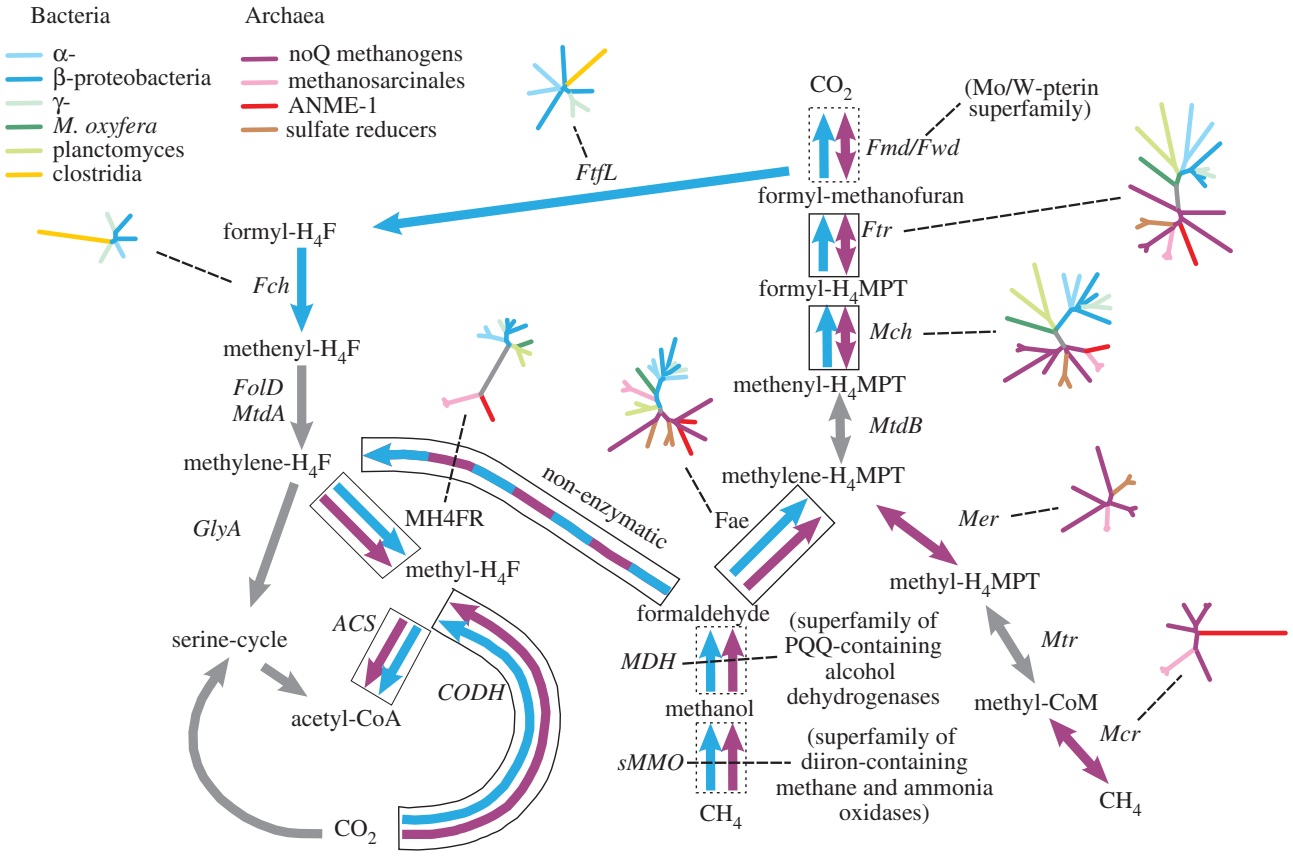

Figure 4. Schematic of the methanotrophic reaction pathways detailed in figure 3 tying together the bacterial and archaeal pathways into a common scheme by means of their shared reaction steps and enzymes. As in figure 1, bacterial and archaeal reaction steps are denoted by blue and violet colours, respectively. For the majority of involved enzymes, phylogenetic trees were reconstructed according to the NJ-algorithm (using MEGA-4). Phylogenetic reconstructions were performed on multiple alignments of sequences retrieved via genomic blast on the NCBI's website (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). The following species were considered: *Methylobacterium extorquens* PA1 (α-proteo), *Methylosinus trichosporium* OB3b (α-proteo), *Methylobacillus flagellatus* KT (β-proteo), *Methylibium petroleiphilum* PM1 (β-proteo), *Methylotenera versatilis* 301 (β-proteo), *Methylococcus capsulatus* str. Bath (γ-proteo), *Methylomonas methanica* MC09 (γ-proteo), Candidatus *Methylomirabilis oxyfera* (NC-10), *Rhodopirellula baltica* SH 1 (planctomyces), *Blastopirellula marina* DSM 3645 (planctomyces), *Clostridium acetobutylicum* EA 2018 (clostridia), *Methanopyrus kandleri* AV19 (methanogen without methanophenazine), *Methanosphaera stadtmanae* DSM 3091 (methanogen without methanophenazine), *Methanosphaerula palustris* E1–9c (methanogen without methanophenazine), *Methanococcus maripaludis* X1 (methanogen without methanophenazine), *Methanococcus vannielii* SB (methanogen without methanophenazine), *Methanosarcina mazei* Go1 (methanosarcinales), *Methanosarcina acetivorans* C2A (methanosarcinales), *Methanosarcina barkeri* str. Fusaro (methanosarcinales), ANME-1 (uncultured methanotrophic Archaeon), *Archaeoglobus fulgidus* DSM 4304 (sulfate-reducing Archaeon), *Ferroglobus placidus* DSM 10642 (sulfate-reducing Archaeon). Schematic versions of obtained trees highlighting affiliation of branches to major phyla via colour coding, as indicated in the top left corner of the figure, are represented next to the respective enzyme. Reaction steps catalysed by enzymes indicated to have been present in the LUCA by their molecular phylogeny are boxed. Dotted boxes denote enzymatic steps for which no reliable phylogeny is available so far but which we consider likely to have operated in the LUCA based on other evidences as discussed in the text. The detailed versions of the phylogenetic trees are available as the electronic supplementary material.

0-state (formaldehyde/methylene). The subsequent oxidation reactions leading up to +4 (CO_2), by contrast, are similar. The ensemble of these observations raises the suspicion that methanotrophy and not aceto- or methanogenesis may have been the founding metabolism in the LUCA. To further assess this, admittedly, iconoclastic scenario, we have reconstructed phylogenetic trees of the enzymes involved in the mentioned three pathways (figure 4). For these trees, only species for which the operation of at least parts of the considered pathways has been conclusively demonstrated were taken into account. To avoid strongly unbalanced trees, we have restricted the number of archaeal methanogens and of proteobacterial methano/methylotrophs to a few representative species from each phylum or subgroup. As indicated in the top left corner of figure 4, the considered species comprise α-, β- and γ-proteobacterial methano-/methylotrophs, the anaerobic methane oxidizer *M. oxyfera*, planctomycetes and acetogenic clostridia within the Bacteria as well as methanogens devoid of the liposoluble carrier methanophenazine (all major phyla), methanosarcinales, sulfate reducers and the anaerobic methane-oxidizers from the ANME-1 group.

Figure 4 schematically shows the reaction steps linking acetogenesis, methanogenesis and methanotrophy. For the majority of involved enzymes, phylogenetic trees were reconstructed and are shown next to the corresponding enzymes. Blue and violet arrows denote reaction steps observed in Bacteria and Archaea, respectively. Boxes surrounding arrows indicate reactions catalysed by enzymes suggested by phylogeny to have been present in the LUCA. For example, the trees reconstructed for the enzymes Ftr, Mch, Fae and MH_4FR show a pronounced cleavage into bacterial and archaeal subtrees, and the respective subtrees correspond well to current species trees. As mentioned at the beginning of this article, phylogenies for ACS and CODH have been reported before [35,36] and are in line with the presence of these enzymes in the LUCA. The condensation of formaldehyde with H_4F to form methylene-H_4F is spontaneous and non-enzymatic and therefore can be safely assumed to have

existed in the LUCA. Formylmethanofuran dehydrogenase (Fmd/Fwd) is a member of the superfamily of molybdopterin enzymes (the so-called CISM superfamily [30]). The presence of several subfamilies in LUCA has been suggested recently based on molecular phylogeny [31]. The specific subfamily Fmd/Fwd, however, has not yet been analysed in detail. As discussed in Schoepp *et al*. [31], phylogenetic analysis of this roughly 1000 amino acids containing enzyme is not trivial and has to await a dedicated study. The di-iron enzyme methane monooxygenase and the enzyme methanol dehydrogenase again are part of large superfamilies [102,103]. Intriguingly, both enzymes display considerable substrate promiscuity. Members of both superfamilies are found in Bacteria and Archaea, and phylogenetic analyses have been reported but are debated. The mentioned substrate promiscuity of both enzymes, however, makes us confident that at least the respective enzymatic activities were part of LUCA's repertoire. Accordingly, these reaction steps are boxed, although with dotted lines to indicate the absence of strong phylogenetic evidence.

By contrast, the enzymes Mer and Mcr were found to be restricted to Archaea, whereas FtfL and Fch were found only in Bacteria. We refrained from presenting results for the enzymes Mtr, MtdA/FolD, MtdB and GlyA for the following reasons: (i) Mtr is the coupling enzyme in methanogens devoid of liposoluble electron carriers and consists of at least eight individual subunits. The proposed architecture of this enzyme [38] suggests to us that it is likely made up from individual building blocks also existing in other enzymes (see [104]). The evolutionary history of Mtr therefore is potentially very complicated as also indicated by our preliminary phylogenetic results. Mtr deserves a dedicated and an exhaustive phylogenetic analysis which is beyond the scope of this article. (ii) Searches for MtdA/FolD, MtdB and GlyA genes came up with a substantial number of ostensible paralogues. Without a more exhaustive analysis, we felt unable to decide whether given pairs of genes corresponded to ortho- or paralogues.

The overall picture emerging from figure 4 is that of a pre-LUCA presence of all essential enzymatic steps involved in methanotrophy via methanol/formaldehyde and an only selective presence of methanogenic as well as the methyl-CoM-mediated methanotrophic pathways in Archaea and the acetogenic one in Bacteria. It is noteworthy that the postulated enzymatic route to the carbon-fixing steps via the branch point formate [87] is not supported as an ancient feature of methanol/formaldehyde-based methanotrophy by figure 4. We were unable to find the enzyme FtfL proposed to be essential for this route, not even in *M. oxyfera*, let alone in Archaea. Figure 4 thus suggests that the formate connection may be a more recent mechanism restricted to clostridia and proteobacteria. The ancestral route would then likely correspond to the direct shunt from formaldehyde to H_4F to yield methylene-H_4F in a spontaneous reaction (of course first requiring a probable enzymatic import of the periplasmically produced formaldehyde into the cytoplasm).

8. If methanotrophy really was so ancient, why do we not find it in many more prokaryotic phyla?

Until a few years ago, methanotrophy did not look an especially good candidate for an ancestral energy and carbon metabolism. It was only observed in a few 'exotic' members of the highest subgroups of the proteobacteria and obligatorily required molecular oxygen. Although the second stepping stone has been removed with the discovery of *M. oxyfera*, methanotrophy still cannot be considered a ubiquitous type of bioenergetic system. Traditional WL systems do not fare much better with respect to this point of criticism. Acetogenesis is a relatively rare trait in Bacteria and methanogenesis, although considered for a long time to be the archetypal energy metabolism in Archaea, increasingly turns into just one of many ways of free energy conversion mastered by the Archaea (figure 2). The perceived similarity of the WL pathways in aceto- and methanogens surely would alleviate the problem of rarity by feigning wide phylogenetic distribution of the WL pathway principle. However, as we have tried to convey at the beginning of this article, the perception of similar WL pathways in aceto- and methanogens appears to turn from commonplace reality into a mirage.

But do we really have to care about wide species distribution for an energy-converting system to qualify as ancient? A plethora of geochemical evidences tell us that our modern planet is a world away from the Earth of the Hadean and the Archaean. This is particularly true when it comes to the electrochemical substrates powering the chemiosmotic machinery. Although prior to the rise of oxygen, alternative oxidants such as sulfate, nitrogen oxides and oxyanions or ferric iron may have been extensively recruited by life for bioenergetic purposes, these electron acceptors and their corresponding metabolisms are now vastly outcompeted by aerobic respiration. Oxygen-respiring species therefore dominate the surviving branches of the tree of life. The majority of species depending on anaerobic types of energy metabolism either died out or made it into increasingly restricted niches devoid of oxygen but rich in the 'ancestral' redox substrate. The major geochemical transitions of our planet thus must have pruned the tree of life, heavily thinning out the branches which conserved energy the 'ancient way'. Sparse occurrence of a given energy metabolism on the tree of life therefore is a requirement rather than an impediment for qualifying as potentially ancient. Neither acetogenesis nor methanogenesis nor even anaerobic methanotrophy therefore needs to be intimidated by the argument frequently raised by the phylogenomics community that rare traits cannot be ancient traits. However, even if they are only sparsely represented on the tree of life, their constituent enzymes do have evolutionary histories that can be reconstructed and it is these histories which should guide our assessment of ancestry, all probabilistic aspects of molecular phylogeny and possible sources of error notwithstanding. Based on the results shown in figure 4 we argue that methanogenesis and acetogenesis do not pass the phylogenetic litmus test for pre-LUCA presence, making anaerobic methanotrophy via methanol and formaldehyde the only remaining candidate at the time being. As discussed in §5, anaerobic methanotrophy furthermore optimally exploits redox and carbon substrates delivered by submarine alkaline hydrothermal systems, our preferred model for life's hatchery.

9. Methanotrophy fulfils the fundamental demands imposed by thermodynamics

We as well as others [3–5,11] have previously proposed that the first metabolic pathways of life represent a 'quickening' of

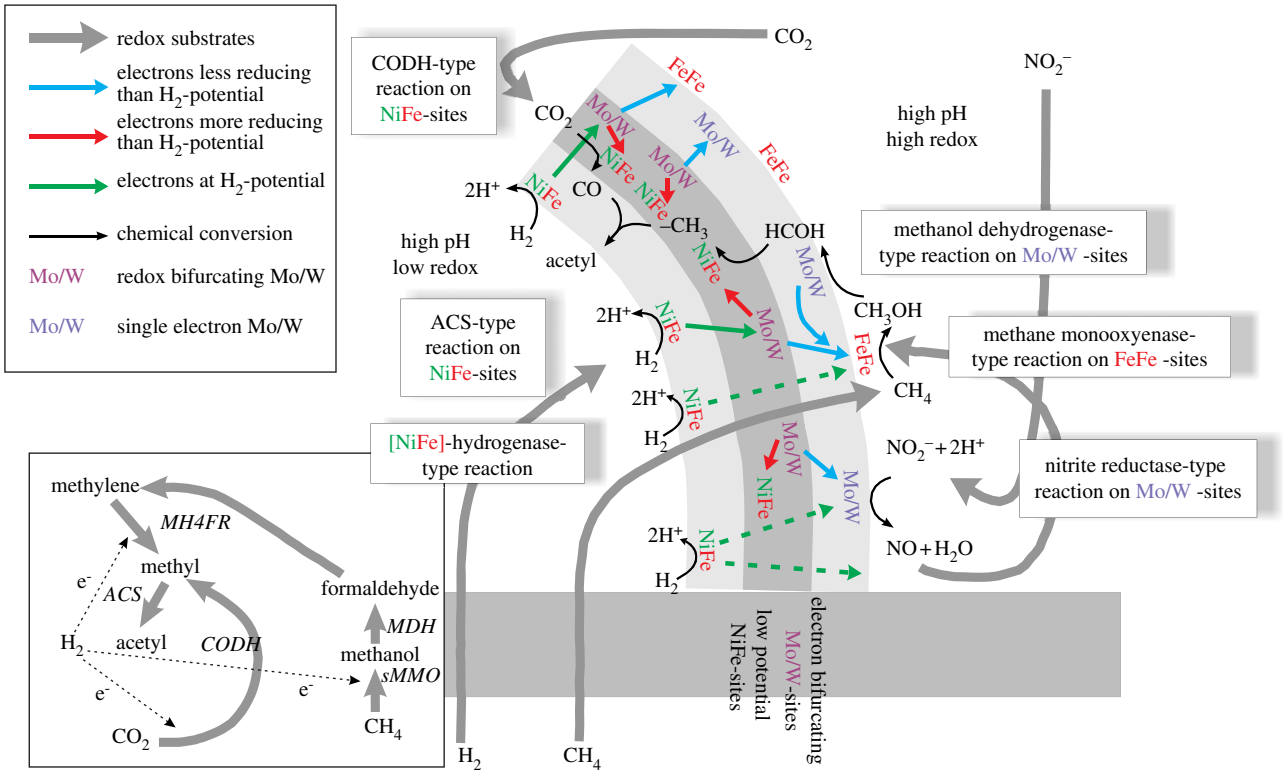

Figure 5. Schematic of a compartment barrier in a hypothetical alkaline hydrothermal seep in the Hadean as developed previously [3] but amended by the additional presence of methane and nitrite. Reducing equivalents are continuously produced on the inner surface by oxidation of H_2 on NiFe-centres. These reducing equivalents flow downhill with respect to the electrochemical potential imposed by the pH gradient, that is, from inside to outside. Only the very low potential electrons produced in redox bifurcating reactions (see text and [108]) can partially move against the 'membrane potential'. H_2 oxidation (releasing protons) and nitrite reduction (requiring protons) are favoured at the inner and outer side of the barrier, respectively, due to the pH gradient. This further increases the free energy content of the composite 'denitrifying methanotrophic acetogenesis' reaction potentially catalysed by the structure shown. The coating of the outer face of the mineral barrier by green-rust-like structures resembling the catalytic sites of di-iron methane monooxygenases of extant methanotrophs is proposed to represent the site of methane oxidation to methanol. Methane oxidation can only occur on the outside due to the likely inability of the charged nitrogen oxyanions to cross the barrier. The stipulated catalytic reactions are inspired by the minimal scheme (inset) of denitrifying methanotrophic acetogenesis as emerging from figure 4.

inorganic reactions, namely the inorganic reduction by H_2 of CO_2 to methane. A rigorous analysis of the thermodynamic requirement for life to emerge [8] has cast serious doubts on the very premises of this way of thinking. The traditional WL reactions with their extremely low free energy yield are in this analysis seen as badly suited for driving the entropy-decreasing engines that turn the origin of life into a necessity rather than an infinitely improbable event. Methanotrophy, in flagrant contrast to aceto- and methanogenesis, does not merely emulate the inorganic reduction of CO_2 to methane but, on the contrary, makes full use of the comparatively enormous free energy exploitable through collapsing the redox disequilibrium between methane, on the one hand, and nitrogen oxides and oxyanions, on the other hand. Moreover, and crucially, in terms of the thermodynamic requirements for life to originate [8,9], methanotrophy is a macroscopic electron-bifurcating device. The oxidation of methane to methanol, pulled ultimately by nitrite, prompts a cascade of redox reactions which, at each step, produce chemicals that are more reducing than those of the preceding steps, a quintessential free-energy-converting device as considered in non-equilibrium thermodynamics [8,105].

Methanotrophy is thus not only predicted as ancestral energy and carbon metabolism by the biological arguments outlined in this article. It also perfectly fulfils the requirements imposed by non-equilibrium thermodynamics and by that it further differs from aceto- and methanogenesis.

We will therefore in the following try to propose a scenario for the founding energy and carbon metabolism of nascent life based on methanotrophy, exclusively relying on inorganic cofactors.

10. A bioinspired scenario for the earliest energy and carbon metabolism

Our basic setting is that of a submarine alkaline hydrothermal vent to which H_2, CH_4, and the essential transition elements, molybdenum and/or tungsten [4], are delivered at high pH from the base of the hydrothermal hatchery through the process of serpentinization [106]. The periphery of this structure is exposed to the acidulous ocean containing high concentrations of CO_2 and sizeable amounts of nitrogen oxyanions which will slowly diffuse into the mound's interior [9]. Mineral membranes [9,51,107] provide pH and redox boundaries at which crucial reactions are catalysed by inorganic metals or metal clusters. This setting is schematically represented in figure 5.

How can an ancestral methanotrophic pathway as inferred from the phylogenetic results detailed in §7 have operated in this setting? The inset of figure 5 illustrates the 'core' of the pathway, that is, all the elements that appear to have been present in the LUCA and that do not appeal to more complicated organic molecules such as the

methanopterins. This core pathway only requires methane, hydrogen and carbon dioxide to form the acetyl-moiety.

The centrepiece of our scenario is molybdenum (or tungsten) atoms, some of which are located in electrostatic environments inducing strongly crossed-over individual redox potentials making these metals obligatory 2-electron redox compounds [108] (denoted by violet characters), whereas others are tuned by the environment to act as single-electron redox compounds (shown in blue in figure 5). We propose that these Mo/W atoms are constantly replenished in reducing equivalents by two-electron reduction from H_2 mediated by NiFe-centres analogous to those in extant [NiFe] hydrogenases (represented by 'NiFe' at the inner surface of the inorganic membrane in figure 5).

The initial trigger for the reaction cascade is provided by the reduction of nitrite to NO at such Mo/W centres. Although in extant species the reduction of nitrite to NO is catalysed either by a haem (haem d_1) or by a binuclear copper centre, the Mo enzyme nitrate reductase has been shown to also have measurable nitrite reductase activity providing proof of principle that this reaction can indeed by achieved by a Mo/W centre.

The resulting NO may bind to di-iron centres such as are present in green rust minerals coating the outer surface of the FeS-based inorganic membranes [9] where the oxygen atom can be activated by electrons which it extracts from a strongly redox crossed-over Mo/W centre. Such a Mo/W centre, however, does only reduce the oxygen if it can simultaneously dispose of its second, more strongly reducing electron towards a suitable acceptor [8,108]. In our scenario, this acceptor is the NiFe-containing mineral greigite ($\sim Fe_5NiS_8$), resembling the cofactor of CO-dehydrogenase [52,109]. To be a suitable acceptor for the second electron, this NiFe site must be in the oxidized state which implies that its midpoint potential is substantially lower than the ambient potential imposed by the flow of H_2. This situation is perfectly analogous to what is observed in extant life where the low redox potential required for reduction of CO_2 to CO is ultimately produced by electron bifurcation on flavin-containing enzymes such as the Rnf complex [34,39,40]. We propose that, in the same way as CODH, the low potential NiFe mineral reduced via Mo-mediated electron bifurcation is able to produce CO from CO_2.

Meanwhile, the methanol resulting from methane oxidation on green rust-like di-iron centres comprising the outer zone of the inorganic membrane will be further oxidized to formaldehyde at a Mo centre resembling that performing nitrite reduction [110,111]. The reducing power of the electrons issuing from this reaction is sufficient (cf. Figure 3) to feed both the initial activation of O_2 on the di-iron centre and the reduction of nitrite to NO. The sequence of the two oxidation reactions from methane to formaldehyde therefore represents a positive redox feedback loop. The formaldehyde molecule may then become attached to the nickel-bearing mineral cluster, mackinawite (FeS) [112]. Once there, it would be further reduced to a methyl moiety as the Fe(Ni)S cluster is oxidized to a violarite ($Fe_2Ni_4S_8$) cluster [52,109]. Adsorbed thus it would eventually be condensed with CO through an ACS-type reaction to an acetyl moiety. The fate of this acetyl moiety may then be equivalent to that proposed in the previous models building on the WL pathway. The mineralogical details of this scenario as well as the thermodynamic ramifications are discussed in the accompanying article [9].

11. From Wood–Ljungdahl back to the origin of life: the denitrifying methanotrophic acetogenic pathway

The scenario we propose builds on the groundbreaking appreciation that reactions in the WL pathway are particularly appealing in the context of metabolism-first type origin-of-life models at hydrothermal settings. As is obvious from figure 4, many aspects of the original WL-type scenario still hold in our model. We consider, in fact, that our rendition is a further development of the original WL scenario taking into account recent data from both geochemistry and biology. The main difference consists in the fact that the WL pathways in aceto- and methanogens are not seen as founding principles but as derived from an even earlier type of biomass-accumulating metabolism which is proposed to have driven life into existence and to have persisted up to the ages of the LUCA. The appearance of aceto- and methanogenic pathways would, in our hypothesis, correspond to recycling and adaptation of parts of the pre-existing methanotrophic scheme to deal with alterations in environmental conditions, i.e. scarcity of methane and/or of nitrogen oxyanion-type electron acceptors. Aceto- and methanogens would thus be species able to survive under the most extreme free energy stress still tolerable by life. This dearth of free energy imposed by the only available redox substrates would have prohibited the use of quinone-based electron transfer chains, and the thermodynamic constraints would thus have restricted chemiosmotic energy conversion to a single step, i.e. the minimum required to sustain life. Forced to abandon the quinone system, acetogens and methanogens would, independently, have been obliged to invent redox-driven but quinone-independent charge-translocating systems; hence, the dissimilar enzymes Rnf and Mtr.

Postulating methanotrophy to have driven the LUCA's energy and carbon metabolisms furthermore naturally rationalizes why quinone-based electron transfer may be ancestral. Assuming the scheme of figure 5 as the founding carbon and energy metabolism, there was plenty of excess electrochemical driving force in between the methane/methanol and the nitrite/NO (and eventually the NO/N_2O) couples which only begged to be harvested. Using quinones as liposoluble hydrogen carriers, indeed, appears the simplest and most versatile solution to the problem of harnessing electrochemical free energy as proton motive force [16]. As suggested by figure 5 and as imposed by plausibility, quinones were added to the methanotrophic scheme only substantially after the exclusively metal-based start-up of the first metabolic engine, and the same can safely be assumed for folates and methanopterins. It is obvious from the scheme of figure 5 that carbon fixation and electron transfer (later to become energy harvesting) were originally hard-wired in a 1 : 1 stoichiometry. The (later) addition of the MPT branch towards CO_2 would have enabled two metabolically crucial innovations: (i) partial uncoupling of energy conservation from carbon fixation and thus tuning of the respective ratios of the two pathways to meet fluctuating metabolic needs of the nascent cell and (ii) providing low potential reducing equivalents (in the form of NADH and/or $F_{420}H_2$) to replace H_2 in a plethora of anabolic reactions.

This 'denitrifying methanotrophic acetogenic' pathway thus encompasses all features required by geochemistry, biology

and, last but not least, thermodynamics to kick-start the inevitable journey to life in alkaline hydrothermal settings [9].

We appreciate help from Elbert Branscomb (Urbana-Champaign/Illinois), Isik Kanik (Pasadena/California), Shawn McGlynn (Pasadena/California), Barbara Schoepp-Cothenet (Marseilles/France), Anne-Lise Ducluzeau (Lincoln/Nebraska), Frauke Baymann (Marseilles/France) and Robert van Lis (Marseilles/France). The research described for this publication was carried out at the Jet Propulsion Laboratory, California Institute of Technology, under a contract with the National Aeronautics and Space Administration with support by the NASA Astrobiology Institute (Icy Worlds) and by the French Agence Nationale pour la Recherche (ANR-Blanc-MC2). U.S. Government sponsorship is acknowledged.

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