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Biochemical fossils of the ancient transition from geoenergetics to bioenergetics in prokaryotic one carbon compound metabolism☆

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ABSTRACT

The deep dichotomy of archaea and bacteria is evident in many basic traits including ribosomal protein composition, membrane lipid synthesis, cell wall constituents, and flagellar composition. Here we explore that deep dichotomy further by examining the distribution of genes for the synthesis of the central carriers of one carbon units, tetrahydrofolate (H_4F) and tetrahydromethanopterin (H_4MPT), in bacteria and archaea. The enzymes underlying those distinct biosynthetic routes are broadly unrelated across the bacterial–archaeal divide, indicating that the corresponding pathways arose independently. That deep divergence in one carbon metabolism is mirrored in the structurally unrelated enzymes and different organic cofactors that methanogens (archaea) and acetogens (bacteria) use to perform methyl synthesis in their H_4F- and H_4MPT-dependent versions, respectively, of the acetyl-CoA pathway. By contrast, acetyl synthesis in the acetyl-CoA pathway — from a methyl group, CO_2 and reduced ferredoxin — is simpler, uniform and conserved across acetogens and methanogens, and involves only transition metals as catalysts. The data suggest that the acetyl-CoA pathway, while being the most ancient of known CO_2 assimilation pathways, reflects two phases in early evolution: an ancient phase in a geochemically confined and non-free-living universal common ancestor, in which acetyl thioester synthesis proceeded spontaneously with the help of geochemically supplied methyl groups, and a later phase that reflects the primordial divergence of the bacterial and archaeal stem groups, which independently invented genetically-encoded means to synthesize methyl groups via enzymatic reactions. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

When it comes to the evolution of bioenergetic systems, the topic of this special issue, of interest is the question of how bioenergetic systems got started in the first place. Clearly, in order to evolve a bioenergetic system consisting of genes, proteins, cofactors and — in the case of chemiosmotic coupling — membranes, there has to be some preexisting, exergonic geological precursor reaction that underpinned the chemical origin of those genes, proteins and cofactors. At some point there was a transition from 'geoenergetics' to bioenergetics, and there hence existed in the very first life forms some core energy releasing reaction that was harnessed so as to allow energy to be conserved in a chemical currency that could be used to promote metabolic reactions that otherwise were sluggish. It would improve our understanding of early evolution immensely to have a better understanding of what that spontaneous geoenergetic reaction was, what the nature of the first bioenergetic reactions was, and the relationship between those two kinds of reactions. Thanks to advances in understanding subsurface energy-releasing chemical reactions that occur in the Earth's hydrothermal systems [4,121], paired with advances in understanding the energetics of anaerobic microbes [26], geochemists and biologists are now finding more common ground for discussion on such questions than ever before. Both sides are talking about redox chemistry, metals, and the exergonic reduction of CO_2 with electrons stemming from hydrogen and iron.

In an early, and insightful, survey of bioenergetics in anaerobes, Decker et al. [37] suggested, based on comparative biochemistry, that methanogens and acetogens are the most ancient forms of energy metabolism among extant microbes: they are strict anaerobes, they tend to lack cytochromes, and they satisfy their carbon and energy needs from the reduction of CO_2 with H_2, substrates that would have been abundant on the early Earth. Forty years later, the basic reasoning behind the idea that anaerobic autotrophs are ancient is still modern [105], it still has many virtues, and the underlying reasons have become much more detailed [56,61,113]. In addition, geological findings independently came to support the antiquity of methanogens because biological methane production was found to go back at least 3.4 billion years [193] and geochemical reactions similar to the core bioenergetic reactions of acetogens and methanogens have been found to occur spontaneously at hydrothermal vents [100,169].

As an alternative to acetate or methane formation, Wächtershäuser [196] suggested that pyrite formation from Fe^{2+} and H_2S was the first source of biological energy. But the pyrite theory did not forge a clear

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Table 1
Comparison of the enzymes that catalyze the different steps of the Wood–Ljungdahl in acetogens and methanogens. Numbers (No.) refer to the steps presented in Fig. 3. Genes, accessions numbers and sequence length are given using the example of *Moorella thermoacetica* and *Methanothermobacter marburgensis* as summarized by Fuchs [61]. The % Identity (I) and % Similarity (S) values refer to the average between all blast hits (see Materials and methods). [a] — average values; * The values correspond to the most similar subunits of the complexes. The line that separates reaction 6 and 12 from reaction 13 was moved relative to Fuchs's [61] notation because the CoFeS small subunit (Moth_1198) also participates in reaction 13.

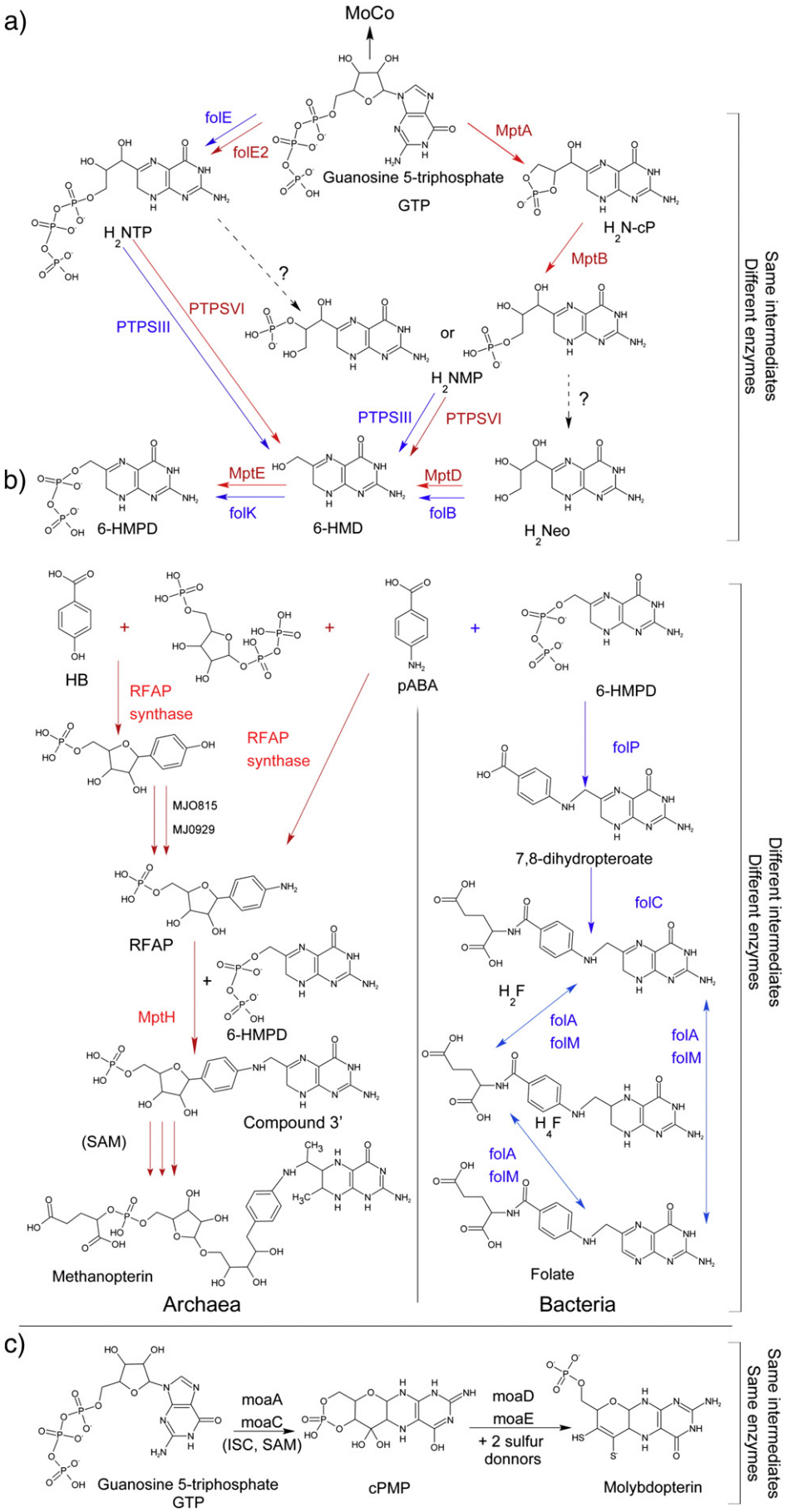
Genes/ accession	Seq. length	No.	Enzyme name	% I[a]	% S[a]	Enzyme name	No.	Genes/ accession	Seq. length
fdhA Moth_2312	899	1	Formate dehydrogenase	11*	19*	Formyl-methanofuran dehydrogenase	7	fmdE MTBMA_c13050	180
								fmdC MTBMA_c13060	400
fdhB Moth_2314	707							fmdB MTBMA_c13070	436
Fhs Moth_0109	559	2	10-Formyl-H_4F synthetase	10	16	Formyl transferase	8	ftr MTBMA_c16460	297
folD Moth_1516	280	3	5,10-Methenyl-H_4F cyclohydrolase/dehydrogenase	11	17	5,10-Methenyl-H_4-MPT (H_4-methanopterin) cyclohydrolase	9	mch MTBMA_c11690	320
		4		12	21	5,10-Methylene-H_4-MPT dehydrogenase	10	mtd MTBMA_c00500	276
metF Moth_1191	306	5	5,10-Methylene-H_4F reductase	13	22	5,10-Methylene-H_4-MPT reductase	11	mer MTBMA_c03270	321
acsE Moth_1197	262	6	Methyl-H_4F: corrinoid iron–sulfur protein methyltransferase	11	18	Methyl-H_4MPT: corrinoid iron–sulfur protein methyltransferase	12	cdh γ/acsC MTBMA_c02920	458
cdh δ/acsD Moth_1198	323		Corrinoid iron–sulfur protein (CFeSP)	25*	40*	Corrinoid iron–sulfur protein		cdh δ/acsD MTBMA_c02910	384
cdh γ/acsC Moth_1201	446	13	CO dehydrogenase/acetyl-CoA synthase	25*	37*	CO dehydrogenase/acetyl-CoA synthase	13	cdh α1 MTBMA_c02870	777
								cdh α2 MTBMA_c14200	395
cdh (α/β)/acsB Moth_1202	729							cdh α3 MTBMA_c14210	311
								cdh α4 MTBMA_c14220	139
cdh δ acsD Moth_1198	323							cdh ε1 MTBMA_c02880	170
								cdh ε2 MTBMA_c14190	169
cdh γ acsC Moth_1201	446							cdh β MTBMA_c02890	460

link to modern microbial physiology, nor did it take into account the vexing ubiquity of chemiosmotic coupling among modern cells [114]. From our standpoint, having a link to modern microbes is important, because very many different possible sources of energy for early biochemical systems can be envisaged, including polyphosphates [12], photochemical ZnS oxidation [125,126], ultraviolet light, and other possibilities [36,91]. But one cannot meaningfully address the ancestral state of microbial energy metabolism among modern forms unless there are organisms known that actually make a living from such sources. The result is that, despite occasional differences of opinion [133,179], there has never been a heated debate specifically about the nature of the first bioenergetic systems. This might, in part, be due to the circumstance that biological energy conservation generally involves quite complicated molecular machines [1] and there exists a bewildering diversity of routes to consider [5,167], such that the question of which one(s) might be the most ancient is thorny.

In contrast to the issue of core bioenergetic reactions, a great deal of attention has been given to the issues of i) whether the first organisms were thermophiles [137,181,182] or not [20,58,62], and ii) and whether they were autotrophs [37,124,197] or not [112,134]. The issue of which pathways they actually used to make a living in the sense of carbon and energy metabolism [97,115] has somehow been of secondary importance. Now is a good time to invigorate the question of the earliest bioenergetic systems, for two reasons. First, newer findings document eyebrow-raising similarities between the bioenergetic reactions of anaerobic autotrophs and geochemical reactions that occur spontaneously at some types of hydrothermal vents [121], an exciting development. Second, electron bifurcation has recently been discovered [104], a mechanism of energy conservation that explains how it is possible for acetogens and methanogens to reduce CO_2 with electrons from H_2, even though the first segment of the reaction sequence is energetically uphill [26]. Electron bifurcation is a major advance in understanding the bioenergetics of anaerobes in general, and of anaerobic autotrophs in particular.

Methanogens and acetogens replenish their ATP pool with a rotor–stator type ATPase that harnesses ion gradients generated during the reduction of CO_2 with H_2 with the involvement of the acetyl-CoA pathway [26]. Among the six CO_2 fixation pathways known, the acetyl-CoA pathway, or Wood–Ljungdahl pathway [107,219], is the only one known that occurs in both archaea and bacteria [16,61]. This and other lines of evidence suggest that it is the most ancient of the six [60,61,114]. In hydrogenotrophic methanogens and acetogens, the acetyl-CoA pathway is simultaneously linked to a pathway of energy metabolism, because these organisms obtain their energy from the reduction of CO_2 to methane and acetate respectively, using H_2 as the electron donor. This is clearly an ancient redox couple for energy metabolism [56,99,105]. In comparisons of the acetyl-CoA pathway in acetogens and methanogens, the use of different cofactors for methyl synthesis from CO_2 stands out: tetrahydrofolate (H_4F) in acetogens versus tetrahydromethanopterin (H_4MPT) in methanogens [49,88,93,109]. The differences in the cofactors are of particular interest because folate is not only central to the acetyl-CoA pathway, it is more generally the universal C1 carrier in bacterial metabolism [118], where it provides C1 units for amino acid, cofactor and nucleotide biosynthesis [109,165,224] in addition to providing the methyl groups for modified bases and ribosome methylation so that the genetic code will work [39,118,180].

In archaea, the situation concerning C1 carriers — a topic that has mostly been investigated in the laboratory of Robert H. White [35,67,201–210,212] — is more diverse, as recently summarized by de


a)
MoCo
folE
folE2
Guanosine 5-triphosphate
GTP
MptA
H2NTP
H2N-cP
MptB
?
PTPSVI
PTPSIII
or
H2NMP
?
PTPSIII
PTPSVI
b)
MptE
MptD
folK
folB
6-HMPD
6-HMD
H2Neo
Same intermediates
Different enzymes
HB
RFAP synthase
RFAP synthase
pABA
6-HMPD
folP
MJO815
MJ0929
7,8-dihydropteroate
folC
RFAP
MptH
6-HMPD
H2F
folA
folM
folA
folM
Compound 3'
(SAM)
H4F
folA
folM
Methanopterin
Folate
Archaea
Bacteria
Different intermediates
Different enzymes
c)
moaA
moaC
(ISC, SAM)
moaD
moaE
+ 2 sulfur donnors
Guanosine 5-triphosphate
GTP
cPMP
Molybdopterin
Same intermediates
Same enzymes

Crécy-Lagard et al. [35], who point out that the archaea, including non-methanogenic forms, generally tend to possess methanopterin or methanopterin-related C1 carriers. Exceptions to this rule are the halophiles, which possess H_4F instead of H_4MPT [19,135], and *Methanosarcina barkeri* strain fusaro, which possesses both H_4MPT and H_4F [25]. Here we examine the phylogenetic distribution of genes involved in H_4F biosynthesis and those known so far in H_4MPT biosynthesis among prokaryotic genomes with the aim of exploring the ancestral state of C1 metabolism in the prokaryote common ancestor.

2. Materials and methods

2.1. Data

Genomes of 1606 prokaryotes (117 archaea and 1489 bacteria) were downloaded from RefSeq database (v03.2012) [152]. Literature searches on the biosynthesis of the different pterins were performed. Homologous proteins involved in the different folate and pterin biosynthesis were identified by BLAST [3] within the data set of downloaded genomes using the proteins from [24,35,46,63,69,70,78,90,103,116,117,144,160,162,172,183,186,187,200,220]. The BLAST lists were filtered for E values better than 10^{-10} and amino acid identities ≥30%. To account for fused genes, the BLAST results were parsed and a gene classified according to its highest similarity hit. If a gene presented the highest homology with a fused one (e.g. *folBK*), the presence of both genes (in this case, *folB* and *folK*) was considered.

Homologous proteins involved in the acetyl-CoA pathway were identified by BLAST [3] within the data set of downloaded genomes using the proteins from [61] and filtered for E values better than 10^{-10} and amino acid identities ≥20% (Table 1). Protein pairs from organisms where the Wood–Ljungdahl pathway is present were globally aligned using the Needleman–Wunsch algorithm with needle program (EMBOSS package) [159].

2.2. Sequence alignments and phylogenetic analysis

Proteins were aligned using MUSCLE [45] using its default parameters. Statistical testing was done using the program SEQBOOT (PHYLIP 3.695 package) [54] by resampling the data sets 100 times. For construction of phylogeny using maximum-likelihood, FastTree 2.1.7 [150] was used with the WAG + G model and four rate categories. A majority extended consensus tree was created with consense (PHYLIP 3.695 package) [54]. Alignments and trees are available upon request.

2.3. Structural analysis

SCOP domain annotations [129] were retrieved by scanning each sequence from Table 1 against the Hidden–Markov Models Library available at the SUPERFAMILY resource [65]. To retrieve the closest available tertiary structure for each family, a BLAST search using the genes from Table 1 as queries was performed against amino acid sequences of protein structures deposited at the Protein Data Bank [17]. The best hits were downloaded and screened for membership in the corresponding protein family according to functional annotation and sequence similarity (no E-value cut-off was employed in order to accommodate differences in substitution rates across sequences). This provided related structures for nearly all of the enzymes numbered 1–13 shown in Fig. 3. That is, there was a structure available in PDB for a protein carrying the same functional annotation as the query, except for i) the B subunit of *Moorella* formate dehydrogenase, ii) the B subunit of *Methanothermobacter* formylmethanofuran dehydrogenase, and iii) the β, δ, and γ subunits archaeal CODH/ACS.

We then compared structures in two steps. In the first step, we asked whether the acetogen and the methanogen enzymes are related by comparing the structures of the proteins corresponding to the acetogen and methanogen enzymes pairwise using DaliLite version 3.1 with the pairwise option [84]. The DALI algorithm uses a weighted sum of similarities of intra-molecular distances to infer structural similarities. Dali-Z scores above 2 are taken as evidence for significant structural similarity in pairwise structural comparisons [84]. Structural alignments were manually checked with Pymol (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

In the second step, we asked: to which protein families in PDB the acetogen and methanogen structures are most closely related. This was done by submitting the coordinates of the PDB entries corresponding to reactions 1–13 in Fig. 4 to a structural similarity search at the DALI server to find its structural homologues. This revealed whether the acetogen and methanogen structures are more similar to each other than they are to other structures represented in PDB or whether they just share common folds. Results are summarized in the Supplemental Material Tables 1 to 15.

3. Results and discussion

3.1. The cofactor pathways

Cofactors play an important role in metabolism, doing most of the necessary catalysis. For example, in some pyridoxalphospate (PLP) dependent decarboxylases, the PLP cofactor can be responsible for enhancing the catalytic efficiency by 10^{10} while the protein itself contributes with just an additional 10^8 fold [223]. The pathways of H_4F, H_4MPT and molybdopterin biosynthesis are shown in Fig. 1. Guanosine-5′-triphosphate (GTP) is the starting point for the synthesis of all pterin branches (Fig. 1). Folate and methanopterin in addition to the pterin moiety also contain an aminobenzoic acid moiety. Moreover, these structural analogs share a common pterin precursor, 6-hydroxymethyl-7,8-dihydropterin diphosphate (6-HMDP) and the presence of a non-pterin group, L-glutamate in case of folate and phosphoribosyl pyrophosphate (PRPP) in the case of methanopterin (Fig. 1A,B).

The bacterial chorismate branch of the pterin pathway entails two sequential steps for the formation of pABA from chorismate. Using the amine group of glutamine as donor, chorismate is first aminated to 4-amino-4-deoxy-chorismate by *pabA* and *pabB* [162] and then converted to pABA by 4-amino-4-deoxychorismate lyase (*pabC*) [69]. Independent events of fusion of these genes occurred and combinations of *pabAB*, *pabBC* or even *pabABC* have been reported [24,200]. Although methanogens can incorporate pABA into methanopterin if provided in the culture medium [205,212], genes from this pathway are absent and instead of pABA, methanogens incorporate 4-hydroxybenzoic acid (HB) into methanopterin [212].

The synthesis of the pterin moiety of methanopterin and folate share a common intermediate 6-HMDP and several routes for its formation are found in both domains [34,35,109] (Fig. 1A). The most well characterized (here called 6-HMPD branch) is the bacterial route where GTP is converted to 6-HMDP by the *folE* (or *folE2*)/*folB*/*folK* route. First, GTP is hydrolyzed to 7,8-dihydroneopterin triphosphate (H_2NTP) by two evolutionary unrelated enzymes, GTP cyclohydrolase IA (*folE*) [222] (Zinc-dependent) and GTP cyclohydrolase IB (*folE2*) (Zinc-independent) [166]. The phosphatases responsible for the removal of the triphosphate motif via a 7,8-dihydroneopterin monophosphate (H_2NMP) intermediate are still unidentified and the resulting 7,8-dihydroneopterin

Fig. 1. Biosynthesis of folate and pterin derivates, redrawn from [35,67,171,186,210]. A) Synthesis of molybdopterin and 6-HMDP (last folate and methanopterin precursor) from GTP. Blue arrows represent the bacterial 6-HMDP branch and red arrows the alternative 6-HMDP archaea branch. Dashed arrows represent steps where no enzyme has been yet identified. B) Folate (blue) and methanopterin (red) biosynthesis from 6-HMDP. C) Molybdopterin biosynthesis from GTP. The black arrows represent the two steps required for molybdopterin biosynthesis common to both prokaryotic domains.

(H_2Neo) is then converted to 6-hydroxymethyl-7,8-dihydropterin (6-HMD) by a 7,8-dihydroneopterin aldolase (*folB*) [81]. Alternatively, pyruvoyltetrahydropterin synthase paralogs *PTPS-III* [41,149] and archaeal-specific *PTPS-VI* [144] can catalyze the direct conversion of H_2NTP (or H_2NMP) to 6-HMD. The last step is the diphosphorylation of 6-HMD to form 6-HMDP by a diphosphokinase (*folK*) [187,188].

Some archaea have an Fe(II)-dependent GTP cyclohydrolase IB (*MptA*) homologue to *folE2* that converts GTP to a cyclic 7,8-dihydroneopterin 2′,3′-cyclic phosphate (cPMP) intermediate [70]. cPMP is subsequently converted to H_2NMP by a Fe(II) dependent-cyclic phosphodiesterase (*MptB*) [116]. Recently, two alternative archaeal specific enzymes (*MptD* and *MptE*) were identified that replace *folB* and *folK* respectively [35]. The route via *folE2*, *PTPSVI*, *PTPSIII*, *MptA*, *MptB*, *MptD*, *MptE* constitutes the alternative 6-HMDP branch.

6-HMDP is the branching point between folate and methanopterin biosynthesis (Fig. 1B). The condensation of 6-HMDP with pABA is performed by a dihydropteroate synthase (*folP*) leading to the formation of 7,8-dihydropteroate [11,178]. A dihydrofolate synthase (*folC*) catalyzes the formation of dihydrofolate (H_2-folate), adding glutamate to 7,8-dihydropteroate at expenses of ATP. Some organisms have a fused *folP-folC* gene [103] while in other cases, the fusion is between *folB* and *folK* [35]. The conversion of H_2-folate to H_4-folate and/or folate is performed by unrelated dihydrofolate synthases (*folA and folM*). The route *folP*/*folC*/*folA* and/or *folM* complete the branch to folate.

In contrast to the folate pathway, in methanopterin biosynthesis (Fig. 1C), the common intermediate 6-HMPD first reacts with a β-D-ribofuranosylaminobenzene-5-phosphate (RFAP) molecule to form 7,8-dihydropterin-6-yl-4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate [212]. RFAP can be synthesized by the condensation of PRPP with either pABA or 4-hydroxybenzoic acid (HB) [44,157,212]. In both cases, the first reaction is catalyzed by a common enzyme, an RFAP-synthase that is one of the indicators of methanopterin synthesis [15,44,136,157,172].

In the first case, RFAP is directly synthesized by the condensation of pABA with PRPP catalyzed by RFAP synthase [44,157]. In the second case, after an initial condensation of PRPP and HB derived from chorismate or 3-dehydroquinate [212], two additional enzymes, MJ0815 (a possible ATP-grasp enzyme) and MJ0929 (a multifunctional adenylosuccinate lyase), are possibly necessary for the complete RFAP synthesis [212], involving the hitherto unique biological conversion of a phenol to an aniline [212].

The condensation between RFAP with 6-HMDP is performed by the dihydropteroate synthase (*MptH* gene)(Early MPT) [67,220]. A series of additional steps (two of them, SAM dependent) are necessary to convert this product into the final H_4MTP although the enzymes responsible for these steps are yet to be identified [67]. The route via RFAP-synthase/MptH constitutes what is called here the early methanopterin branch.

In the biosynthesis of molybdopterin, the first step consists in the conversion of GTP into the stable cyclic pyranopterin monophosphate (cPMP) intermediate (Fig. 1C) [171]. The formation of the four-carbon atoms of the pyrano-ring by the insertion of the C8 atom of the purine base between the 2′ and 3′ ribose carbon atoms is catalyzed by *moaA* and *moaC* via a S-adenosyl methionine (SAM)-dependent radical mechanism [76,77]. The previously adenylylated (by *moeB* [101]) molybdopterin synthase complex (*moaD* and *moaE*) catalyzes the synthesis of the molybdopterin enedithiolate by incorporating two sulfur atoms into cPMP [214]. Despite the name, molybdopterin itself lacks metal ions. Those are only incorporated in a later stage of the MoCo pathway [171]. The molybdopterin branch consists of the *moa*/*moaC*/*moaD*/*moaE* route.

3.2. Distributions of pathways across genomes

The genes involved in the different pterin biosynthetic pathways were identified in our data set of 1606 complete sequenced genomes (see Materials and methods). Fig. 2 shows the distributions of the genes for molybdopterin, H_4F and H_4MPT biosynthesis for bacterial and archaeal groups. Each column corresponds to a gene and each line to a taxonomic group. The genes are organized by the five different pathways above mentioned (alternative 6-HMPD branch, early MPT branch, 6-HMPD branch, folate branch, and molybdopterin branch). The dots within each row are colored according to the proportion of organisms within the taxon that have the gene coding for the enzyme. Genes for the molybdopterin biosynthesis are widely distributed among both prokaryotic domains and it has remained highly conserved also in eukaryotes [199].

For most of the enzymes involved in the synthesis of the common 6-HMDP intermediate of folate or methanopterin biosynthesis, a domain specific pattern is observed. For instance, although different routes for the synthesis of 6-HMDP within archaeal organisms can be found, for example *folE2*/*PTPSVI*/*MptE* in thermococci, *folE*/*PTPSVI*/*MptE* in sulfolobales, or *MptA*/*MptB*/*MptD*/*MptE* in methanogens, in the present sample, the typically bacterial *folE*/*folB*/*folK* route is only present in a few sulfolobales and one member of the thermoproteales. Even in these cases, a fusion between the *folB* and *folK* has occurred and the genes possibly result from a recent gene acquisition from bacteria [35]. Within bacteria, the opposite picture emerges, with only a few deferribacteres, thermotogae and deltaproteobacteria representatives having genes that allow a partial synthesis of 6-HMDP via the archaeal route.

Comparing the early MPT branch with the two folate branches, this pattern is even more pronounced. Most archaeal organisms have the RFAP synthase responsible for the condensation of RFAP with pABA or HB, while in bacteria the *folP*/*folC*/*folA* or *folM* route is the preferential one. The exceptions among archaea are haloarchaea and thermoplasmatales, which are probably due to lateral gene acquisition from bacteria [132]. There are also gene transfers for H_4MPT biosynthesis into bacteria, for example in the case of the proteobacterium *Methylobacterium extorquens*, where H_4MPT is used for methanol oxidation [29,74].

3.3. Deep divergence and independent origins of unrelated genes

The genes for biosynthesis of molybdopterin are universally distributed within bacteria and archaea suggesting that they were present in their common ancestor. In contrast, there is a clear separation of the distribution of the genes from the folate and the methanopterin pathways. The H_4MPT synthesis genes appear to be conserved throughout all archaea, where those for H_4F are rare and sparsely distributed. Conversely, H_4F synthesis is present in bacteria, where methanopterin synthesis is lacking or rare, as shown in Fig. 2.

Notably, as can be seen in Figs. 1 and 2, many enzymes of pterin synthesis that lead to the same end product are the result of independent evolutionary processes. The parallel origin of enzymes that are i) ancestral for archaea and bacteria respectively, but ii) different in the two groups suggests that when the enzymes arose, they were selected to accelerate preexisting, similar and spontaneous reactions that predate the enzymes themselves. That is, it suggests that the basic underlying chemistry of the pathway is older than the enzymes that catalyze it. That is another way of saying that enzymes do not perform feats of magic, they just accelerate and add specificity to reactions that tend to occur anyway. After all, prior to the origin of genes and proteins, the chemical reactions that led to the origin of translation were by necessity spontaneous and/or catalyzed by compounds in the environment. Thus there had to be chemical reactions taking place continuously in the environment before genes arose.

At face value, there are three possible interpretations for the foregoing observation that archaea and bacteria differ in their C1 metabolism: i) The common ancestor of prokaryotes was archaeal in C1 metabolism and the common ancestor of bacteria reinvented C1 metabolism by evolving the genes for folate synthesis, thereby replacing the preexisting

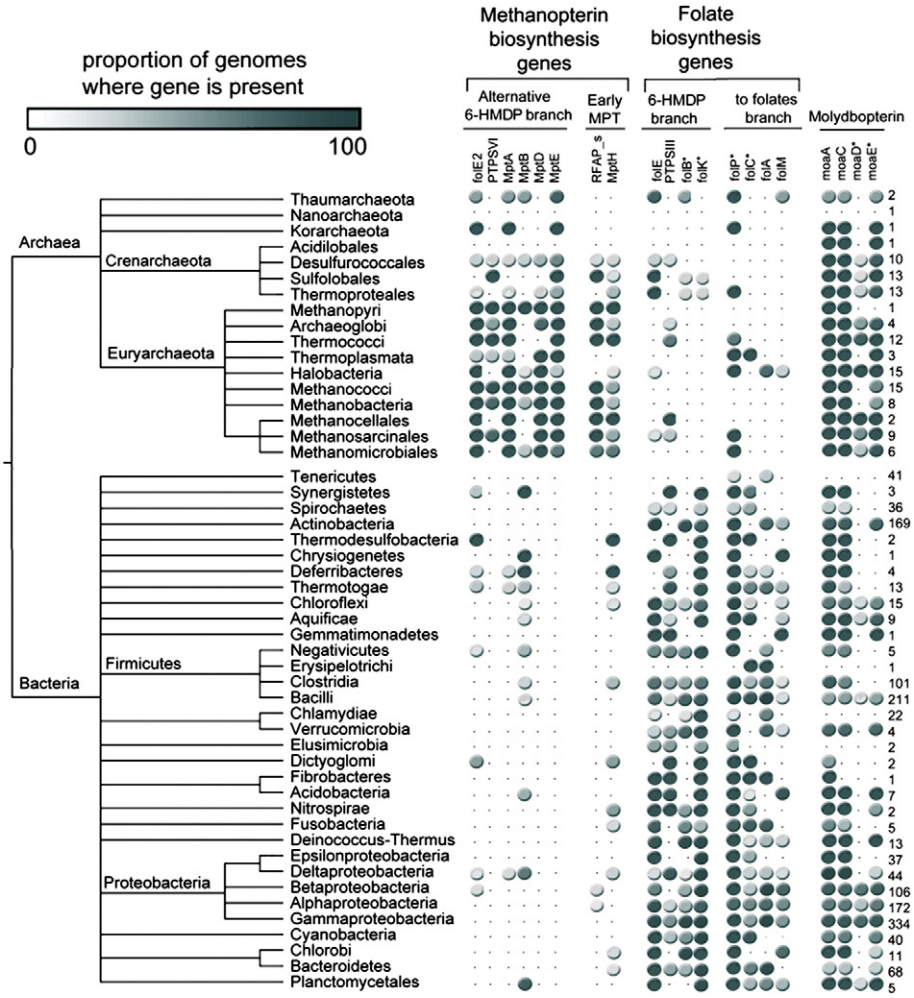


Fig. 2. Distribution of the genes involved in folate and pterin biosynthesis among 1606 prokaryotic genomes. The left part of the figure represents the organization of the selected taxonomic groups from 1606 completed sequenced genomes (117 archaeal and 1489 eubacterial). The right part of the figure represents the proportion of genomes within a taxon where the gene is present. Each column represents a different gene. Homologous proteins involved in the several steps of, the alternative 6-HMDP branch (*folE2*, *PTPSVI*, *PTPSIII*, *MptA*, *MptB*, *MptD*, *MptE*), Early MPT (*RFAP_s*, *MptH*), bacterial 6-HMDP branch (*folE*, *folB*, *folK*), 6-HMDP to folates branch (*folP*, *folC*, *folA*, *folM*) and molydbopterin (*moaA*, *moaB*, *moaC*, *moaE*) biosynthesis pathway were identified by BLAST. *—Some of the genes are fused together. The BLAST results were filtered for E values better than 10^{-10} and amino acid identities of at least 30%. The genes of molybdopterin biosynthesis are widely distributed within the two considered domains. On the contrary, there is a clear separation of the distribution of the genes from the folate and the methanopterin pathways. Note that sequences similar to the bacterial genes for pABA synthesis are found in almost all archaea, but biochemical evidence indicates that methanogens, as a prominent archaeal group, do not synthesize pABA by the bacterial route [148,212].

methanopterin pathway, and the repertoire of enzymes in the bacterial lineage that previously interacted with methanopterin C1 carriers were able to accommodate the new cofactor. ii) The common ancestor of prokaryotes was bacterial in C1 metabolism and the common ancestor of archaea reinvented C1 metabolism so as to replace the folate pathway, the converse scenario to (i) above. iii) The common ancestor of the prokaryotes had neither the folate nor the methanopterin biosynthetic pathway and its C1 metabolism was therefore more primitive, operating with C1 carriers that were spontaneously synthesized in the environment, or with C1 moieties that were spontaneously synthesized in the environment, or both. Scenarios (i) and (ii) have the problem that the gradual de novo evolution of a new and independent C1 carrier pathway in the presence of an existing one does not seem very likely. Scenario (iii) might seem radical, because it implies the existence of C1 carriers and/or chemically accessible C1 units before the advent of genes and proteins. But it is clear that neither genes nor proteins could have arisen without a preexisting, continuous and spontaneous synthesis of C1 units underpinning the synthesis of the building blocks for both.

Scenario (iii) furthermore implies the existence of a common prokaryotic ancestor that was not too different from the progenote that Woese and Fox [217] originally had in mind, namely a non-free-living entity that had a primitive ribosome and the genetic code, but not much more than that in terms of supporting biosynthetic pathways. However, the progenote was originally also seen as the direct progenitor of eukaryotes [216–218]. Subsequent findings indicated the presence of mitochondria in the eukaryote common ancestor [47,127,173,176,195] and the branching of eukaryote informational genes from within the archaeal lineage, rather than as a sister to it [33,92,215], such that today, eukaryotes are more readily understood as having arisen via symbiosis between fully-fledged prokaryotes, with the energetics of mitochondria having played the critical role in that transition [98]. In that sense, the progenote is better seen from today's standpoint as the common ancestor of bacteria and archaea only (reviewed in [215]). A progenote that was depauperate in metabolic genes does not seem unreasonable from the standpoint of genomes, given the very few genes that are universal to all prokaryotes [95,179]. Clearly, there was a phase in early evolution during which an ancestral stock of genes and proteins was being invented (the dawn of enzymes), and it follows that 'some' of those inventions occurred subsequent to the divergence of the ancestral bacterial and archaeal lineages from the progenote. The question of how many 'some' are, and which pathways were affected remains unanswered here. Folate and methanopterin biosynthesis genes appear to belong to that class. Our findings with regard to H_4F and H_4MPT biosynthesis gene differences are

based on a broader sample than that available to Maden [109], who came to a very similar conclusion, namely that "*there are few, if any, close homologues to enzymes of folate biosynthesis among Archaea that utilize H_4MPT, even in the early part of the pathway.*"

As an alternative to the present interpretations, a popular theory has it that many of the molecular differences between archaea and bacteria are the result of adaptation to "energy stress" in the archaeal lineage [194]. The present data on C1 carriers are distinctly at odds with that

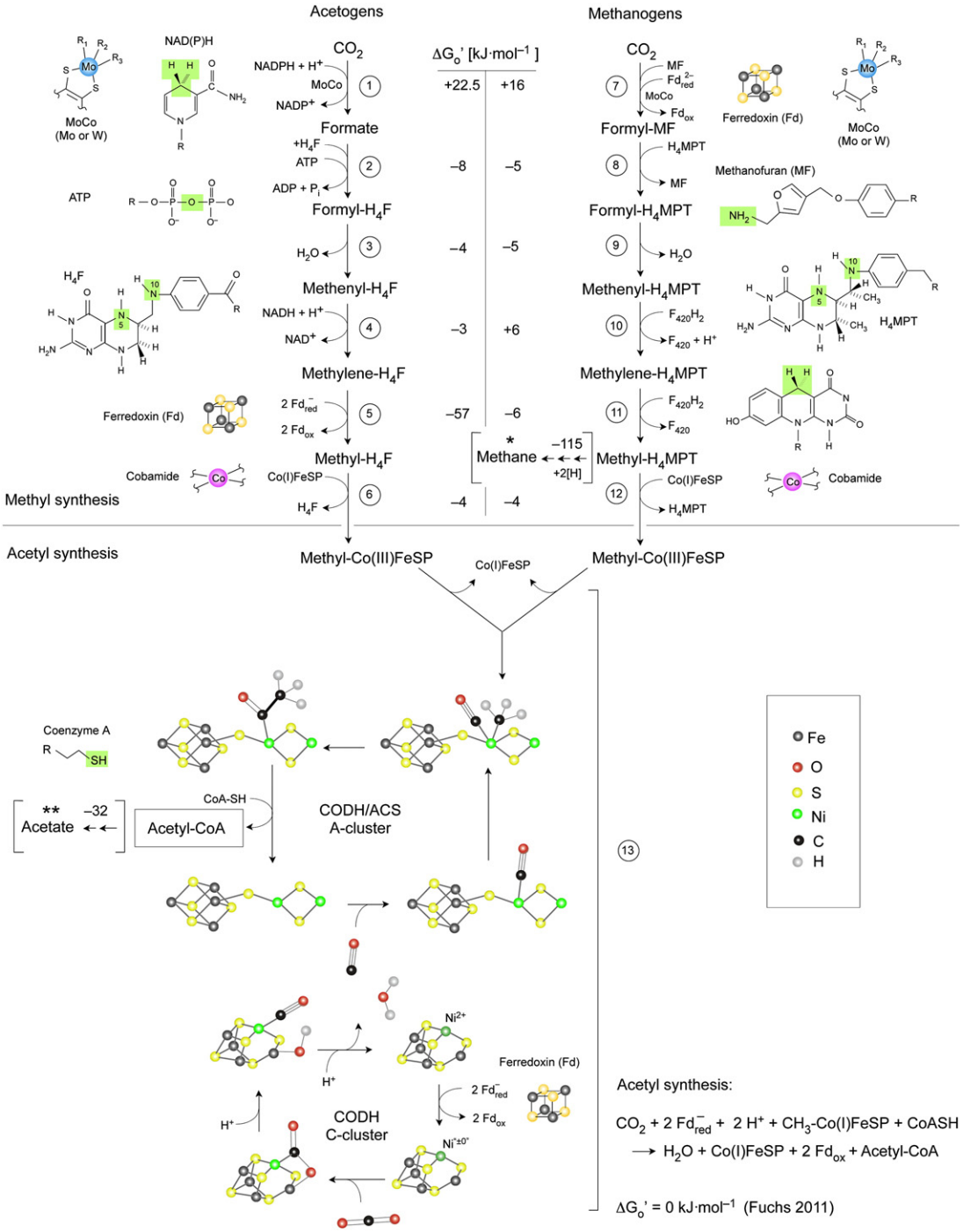


Fig. 3. The Wood–Ljungdahl pathway in acetogens and methanogens using the example of *Moorella thermoacetica* and *Methanothermobacter marburgensis*. The information underlying the figure is redrawn from Fuchs [61] (enzymatic steps, required cofactors, thermodynamic values), from DiMarco et al. ([40]: cofactors), from Graham and White ([67]: structures of the cofactors), and from Ragsdale ([156], supplemental material thereof: simplified reaction mechanism for CODH and ACS reactions). Circled numbers refer to the enzyme names given in Table 1. Abbreviations not explained in the figure are: MoCo, molybdenum pyranopterin; F_{420}, deazaflavin factor 420; NAD(P)H: nicotinamid:adenine dinucleotide (phosphate); H_4F, tetrahydrofolate; H_4MPT, tetrahydromethanopterin; CODH, carbon monoxide dehydrogenase; ACS, acetyl-CoA synthase; Co(I/III)FeSP, corrinoid iron sulfur protein containing Co^{+1} or Co^{+3} respectively. The reactive moieties of the cofactors are shaded with green boxes. It is of interest that the CO_2-reducing reactions of the acetyl CoA pathway involve metal-mediated (Ni, W, Mo) two electron reactions [7]. The asterisk at methane indicates that methanogens harness their energy via reactions involving the methyl group, an exergonic methyl transferase reaction is coupled to sodium pumping [191]. The double asterisk indicates that acetogens synthesize one ATP from acetyl-CoA via acetyl phosphate [146], but this only compensates for the ATP investment at reaction 2; net ATP synthesis in acetogens is dependent upon chemiosmotic coupling and comes from the RNF complex that couples ferredoxin-dependent NADH reduction to sodium pumping [18]. In both acetogens and methanogens, the reduced ferredoxin used during CO_2 fixation is has to be of low potential (ca. −500 mV) because of the low standard potential needed to reduce CO_2, but the electrons needed to generate that low potential Fd_{red} come from environmental H_2, with a standard midpoint potential of about −414 mV. This seemingly uphill reaction is possible because of electron bifurcation [26].

theory. Acetogens live from an even lower free energy change than methanogens [38,190], hence if energy stress was influencing the nature of the cofactors, acetogens and methanogens should use the same type of cofactors and organisms with less energy stress would use different ones. Not so. The differences in C1 carrier synthesis reflect phylogenetic divergence of the lineages that invented folate and methanopterin biosynthesis, not convergent adaptation in response to energy stress.

3.4. More than cofactors

The manifestations of the acetyl-CoA pathway in methanogens and acetogens differ in far more features than just their C1 carriers alone (Fig. 3). In the main, the acetyl-CoA pathway involves two segments: methyl synthesis and acetyl synthesis [61,155,156]. Fig. 3 is redrawn from Fuchs [61] in such a way as to underscore the differences in the bacterial and archaeal cofactors as stressed by White [67], and to include mechanistic aspects of acetyl synthesis as summarized by Ragsdale [156] and Appel et al. [7]. Though labeled "acetogens" and "methanogens", the figure summarizes the data from *Moorella thermoacetica* and *Methanothermobacter marburgensis* [61]. The methyl synthesis branches are very different in acetogens and methanogens and require a number of organic cofactors, while the acetyl synthesis segments are highly conserved and entail almost exclusively inorganic (transition-metal) catalysis.

The first step of methyl synthesis in acetogens entails NADPH-dependent formate dehydrogenase, a Mo- or W-utilizing enzyme, while the first step of the methanogen pathway involves formylmethanofuran dehydrogenase, which requires MoCo, ferredoxin and methanofuran as cofactors. Only the MoCo-binding domain of these two enzymes are related at the level of sequence similarity, and none of the remaining enzymes of the methyl synthesis segment of the pathway are sequence related in comparison of acetogens and methanogens (Table 1). This is true for formyl-pterin synthesis (steps 2 vs. 8, numbered in the figure), the cyclohydrolase reaction that gives rise to the pterin bound methenyl moiety (steps 3 vs. 9), its reduction to a methylene bridge, NADH-dependent in acetogens, dependent on cofactor F_{420} (a flavin relative) in methanogens (steps 4 vs. 10) or the reduction to the level of methyl-pterin that is ferredoxin dependent in acetogens but F_{420}-dependent in methanogens (steps 5 vs. 11). The foregoing reactions are all catalyzed by proteins that lack sequence similarity and have different size and subunit composition (Table 1). Only the MoCo binding subunits of the first step are related at the level of sequence similarity using BLAST [83].

The proteins that catalyze the methyltransferase reactions — steps 6 and 12, encoded by Moth_1197 in *Moorella* and MTBMA_c02910 and MTBMA_c02910 in *Methanothermobacter* — share no readily detectable sequence similarity using BLAST, but they are related at the level of structure by virtue of sharing a common TIM-barrel fold [155]. These methyltransferases connect the methyl synthesis segment with the acetyl synthesis segment in two ways, functionally by transferring the methyl moiety, but also structurally in that the activity is a separate protein in *Moorella* and part of CODH/ACS complex in *Methanothermobacter*. This methyltransferase activity transfers the methyl moiety of methyl-H_4F or methyl-H_4MPT to a cobinamide-bound cobalt that in *Moorella* is the prosthetic group of the large subunit of the CoFeS protein, but in *Methanothermobacter* is a prosthetic group of CODH/ACS. In *M. barkeri*, the methyl-tetrahydropteridine: cob(I)amide methyltransferase (reaction 12) is an activity of the δ-subunit of CODH/ACS [68], by similarity and sequence conservation of methanogen CODH/ACS in *Methanothermobacter* [61] as well. These proteins share a TIM-barrel fold [155]. For clarity, *Moorella* reaction 6 is catalyzed by MeTr ([61,225]). We say "for clarity" because a second methyltransferase activity transfers the methyl group from the cobalt atom in methyl-cobinamide to a nickel atom at the A-site of CODH, a rare metal-to-metal methyl transfer reaction [185], this activity is provided by CODH/ACS itself (Holger Dobbek, personal communication).

In stark contrast to methyl synthesis, the acetyl synthesis segment of the pathway (step 13) is catalyzed by the highly conserved and highly homologous subunits of CODH/ACS in acetogens and methanogens (Table 1). Also in stark contrast to the methyl synthesis segments, which entail five different organic cofactors each in acetogens and methanogens, all of the catalysis in the acetyl synthesis segment is provided by metals, Ni and Fe (Fig. 3). The mechanism of the CODH/ACS reaction, which is redrawn here from Ragsdale [156], involves the Fd-dependent reduction of CO_2 to CO at the C-Cluster of CODH, CO binding to Ni at the A-cluster of the ACS subunit, methyl transfer to Ni from CoFeSP, carbonyl insertion to form the Ni-bound acetyl group, and thiolytic removal from the enzyme via the mercapto group of coenzyme A to yield acetyl-CoA.

3.5. Structural comparisons

The example of the methylpterin:CoFeS methyltransferase, steps 6 and 12, where sequence similarity is lacking but structural similarity is present ([155,225]), is a prescient reminder that the structural features of homologous proteins are more conserved than their amino acid sequences [30,141]. However, shared structural similarity can either indicate an orthologous kind of common ancestry for two proteins (they are each others' closest relatives at the level of structural similarity), or it can also indicate paralogous common ancestry, attributable for example to a shared common fold between two distantly related and distinct protein families. We thus undertook a search for similarities among enzymes of the Wood–Ljungdahl pathway at the level three-dimensional protein structures. Using the available structures for enzymes from the *Moorella* and *Methanothermobacter* pathways, or the structures for those proteins most closely related to them, structural comparisons were performed (see Materials and methods). The results are summarized in Fig. 4.

We start the comparison between steps 1 and 7 (Fig. 4a). *Moorella* fdhA identified *Escherichia coli* fdhH (PDB ID: 1AA6; [21]). *E. coli* fdhH has a four domain αβ structure containing selenocysteine, two molydbopterin cofactors and one Fe_4S_4 cluster. The *M. thermoacetica* sequence has 4 SCOP domains [129] belonging to the superfamilies 2Fe–2S ferredoxin-like, 4Fe–4S ferredoxins, formate dehydrogenase/DMSO reductase domains 1–3 and ADC-like (β-barrel domain). Like *Moorella* fdhA, *E. coli* fdhH finds, by sequence similarity [83], a domain of *Methanothermobacter* fmdB, which belongs to the formate dehydrogenase/DMSO reductase domains 1–3 SCOP superfamily (thick dotted arrow in Fig. 4a). There are no structures available for fmdB. The *Methanothermobacter* fmdC sequence has two domains belonging to the SCOP superfamilies α subunit of glutamate synthase, C-terminal domain and the ADC-like proteins. FmdC, the molybdenum containing protein, shares 35% amino acid sequence identity to the tungsten-containing enzyme, fwdD. The *Archaeoglobus* fwdC (PDB ID: 2KI8) structure is more similar to the VCP-like ATPase (Z-score of 8.8, rmsd 2.8, 99/185 amino acids) than it is to fdhH (Z-score of 7.6, rmsd 15.9, 108/146 amino acids), as indicated by the black arrow to VCP-like ATPase versus the gray arrow to fdhH (Fig. 4a). FwdE from *Desulfitobacterium hafniense DCB-2* (PDB ID: 2GLZ) defines the FwdE-like SCOP superfamily [10] but it has no significant structural hits in PDB to other protein families using DALI. There are currently no structures in PDB for *Moorella* fdhB proteins. By sequence search using MOTH_2314 as query in PDB, the best hits were with the small chain of glutamate synthase (E-value 10^{-43}) from *Azospirillum brasilense* (PDB ID: 2VDC chain G; [32]), and with dihydropyrimidine dehydrogenase (E-value 10^{-28}) from *Sus scrofa* (PDB ID: 1GT8; [42]). The *Moorella* sequence has 4 SCOP domains belonging to the superfamilies Nqo1C-terminal domain-like, nucleotide-binding domain, and alpha-helical ferredoxin (twice). Thus, beyond the homologous MoCo-binding

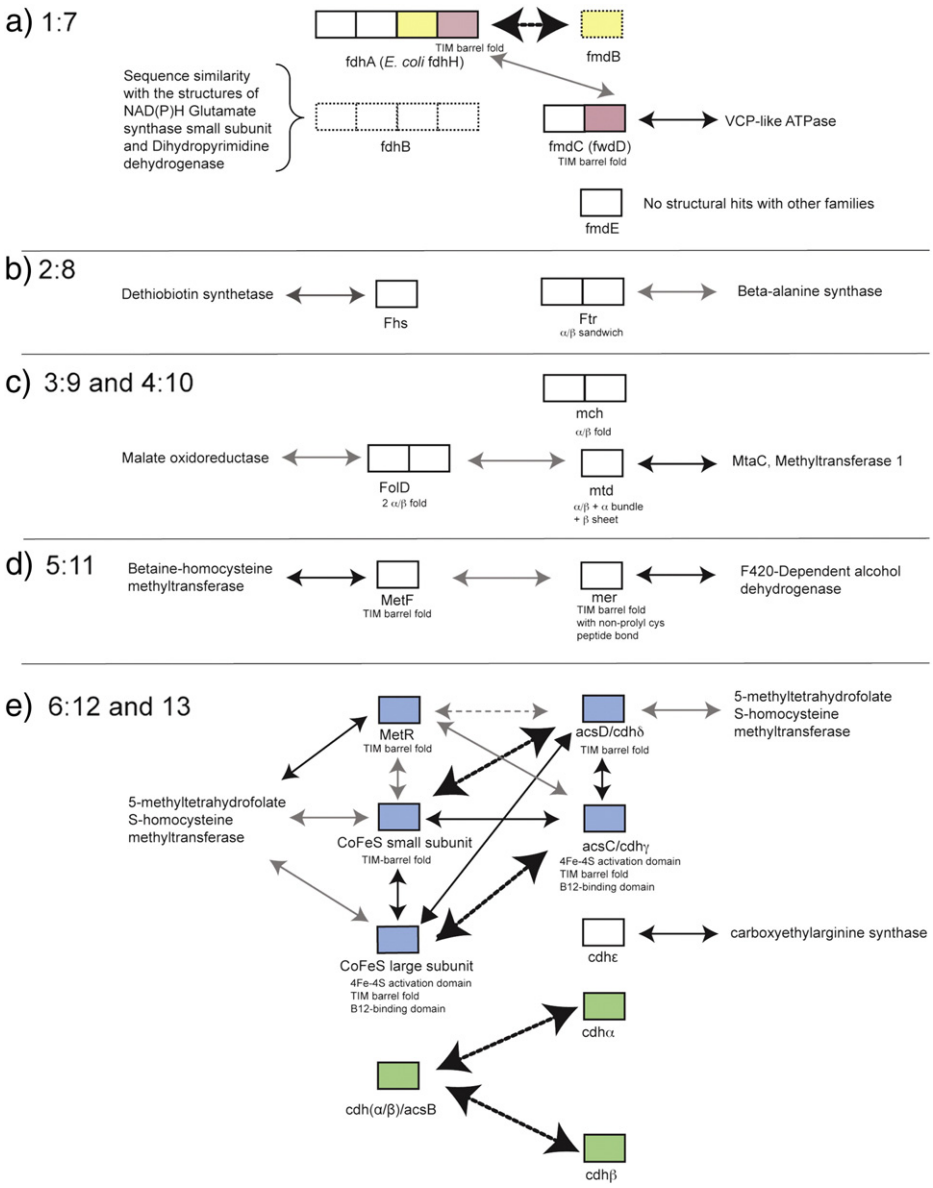


Fig. 4. Structural homology and domain organization between acetogenic (left) and methanogenic enzymes (right) enzymes from the acetyl-CoA pathway as defined in Table 1. Enzymes participating in reactions 1 to 13 given in Table 1 are represented by a full line rectangle if tertiary structure is available and by a dotted rectangle in the cases where there is no available tertiary structure information for that family of enzymes. Each rectangle is divided according to their different SCOP superfamilies domains determined at the SUPERFAMILY server [65]. Similar colors between domains indicate that they belong to the same SCOP superfamily. The similarity between enzymes is represented by arrows: thick dotted black arrows indicate that there is both structural and sequence similarity between the enzymes, black full arrows represent the highest structural similarity and gray full arrows represent lower structural similarity as determined by the DALI structural comparisons ([84]; see Methods). a) Comparison between the Formate dehydrogenase (fdhA, fdhB) and Formyl-methanofuran dehydrogenase (fmdB, fmdD, fmdE) enzymes from reactions 1 and 7. The *Moorella* fdhA is structurally represented by *E. coli* fdhH and the *Methanothermobacter* by the A. fulgidus *fwdD* enzyme. b) 10-Formyl-H_4F synthetase (Fhs) and Formyl-methanofuran dehydrogenase (Ftr) enzymes from reaction 2 and 8. c) 5,10-Methenyl-H_4F cyclohydrolase/dehydrogenase (FolD) and 5,10-Methenyl-H_4MPT cyclohydrolase (mch) enzyme from reaction (3 and 4) and 9 and 5,10-Methylene-H_4MPT dehydrogenase (mtd) from reaction 10. d) 5,10-Methylene-H_4F reductase (MetF) and 5,10-Methylene-H_4MPT reductase from reactions 5 and 11. e) Methyl-H_4F: corrinoid iron–sulfur protein (MeTr), Corrinoid iron–sulfur protein (CFeSP) (CoFeS small and large subunit), CO dehydrogenase/acetyl-CoA synthase (cdh (α/β)/acsB) and the methanogenic acetyl-CoA synthase complex (cdhδ/acsD, cdhγ/acsC, cdhε, cdhα, cdhβ) from reaction 6, 12 and 13.

domains, the acetogen and methanogen enzymes share a common fold, but are not structural orthologues.

For reactions 2 and 8 (Fig. 4b), formyl-H_4F synthetase, Fhs from *M. thermoacetica* [154] (PDB ID: 1EG7), belongs to the P-loop containing nucleoside triphosphate hydrolases SCOP superfamily (Nitrogenase iron protein-like family). The structure of formylmethanofuran:H_4PMT formyltransferase from *Methanopyrus kandleri*, Ftr (PDB ID: 1FTR) has an α/β sandwich fold and the 2 domains define the SCOP family formylmethanofuran:H_4PMT formyltransferase [111]. The two structures show no structural similarity (Z-score below 2) but they each find structural homologs in PDB (Fig. 4b). Thus, the methanogen and acetogen enzymes are structurally unrelated.

Turning to reactions 3 and 4, the FolD enzyme of *Moorella* is bifunctional. For reaction 3, the methylene-H_4F cyclohydrolase domain of *Moorella* found FolD from *Campylobacter jejuni* (PDB ID: 3P2O), as did reaction 4, catalyzed by the methylene-H_4F dehydrogenase domain. FolD has 2 α/β fold domains belonging to the aminoacid dehydrogenase-like SCOP superfamily in the N-terminal domain and NAD(P)-binding Rossmann-fold domains aminoacid dehydrogenase-like, in the C-terminal domain. The cyclohydrolase domain (reaction 3) detects significant structural similarity to malate oxidoreductase (Z-score 16.2, rmsd 3.1, 217/373 amino acids) but no significant structural similarity to the methenyl-H_4MPT cyclohydrolase, mch (PDB ID: 1QLM), from *M. kandleri* [66], which catalyzes reaction 9 and has two domains with a

new α/β fold, defining the methenyl-H_4MPT cyclohydrolase SCOP superfamily (Fig. 4c). For reaction 4, the methylene-H_4F dehydrogenase domain does share low pairwise structural similarity (Z-score 5.2, rmsd 3.4, 102/282 amino acids) to the methylene-H_4MPT dehydrogenase (reaction 10) from *M. kandleri*, Mtd (PDB ID: 1QV9), which has an α/β domain followed by an α-helix bundle and a short β-sheet [73] and defines the F_{420}-dependent methylene-H_4MPT dehydrogenase SCOP family. But Mtd shares much greater structural similarity (Z-score 10.7, rmsd 2.7, 121/258 amino acids) to the MtaC methyltransferase SCOP superfamily than it does to the folate dependent enzyme (Fig. 4c). Thus, in the comparison of reactions 3 and 9, the acetogen and methanogen enzymes are structurally unrelated, while in the comparison of reactions 4 and 10, the acetogen and methanogen enzymes share a common fold but are not structural orthologs.

For reactions 5 and 11 (Fig. 4d), the *Moorella* methylene-H_4F reductase returned MetF from *Thermus thermophilus HB8* (PDB ID: 1 V93). The MetF structure contains a TIM-barrel and belongs to the FAD-linked oxidoreductase SCOP superfamily. The corresponding methanogen enzyme, methylene-H_4MPT reductase from *Methanothermobacter thermautotrophicus*, Mer (PDB ID: 1 F07) has a TIM barrel fold with a non-prolyl cys peptide bond [174] and belongs to the bacterial luciferase-like (F_{420} dependent oxidoreductase) SCOP superfamily. Structurally, MetF is just as similar to betaine homocysteine methyltransferase (Z-score 15.8, rmsd 3.5, 238/348 amino acids) as it is to Mer (Z-score 15.7, rmsd 3.6, 225/321 amino acids), but Mer is much more similar to F_{420}-dependent alcohol dehydrogenases (Z-score 34, rmsd 2.4, 293/330 amino acids), F_{420}-dependent glucose-6-phosphate dehydrogenases and other members of the bacterial luciferase family [9] than it is to MetF. It should be recalled that in another acetogen, *Acetobacterium woodii*, which in contrast to *Moorella* lacks cyctochromes, a different type of methylene-H_4F reductase exists [85] that is clustered in the genome next to a flavoprotein and an RnfC subunit [146] that is lacking in *Moorella*. Because of the very exergonic nature of the methylene-H_4F reductase reaction, it has

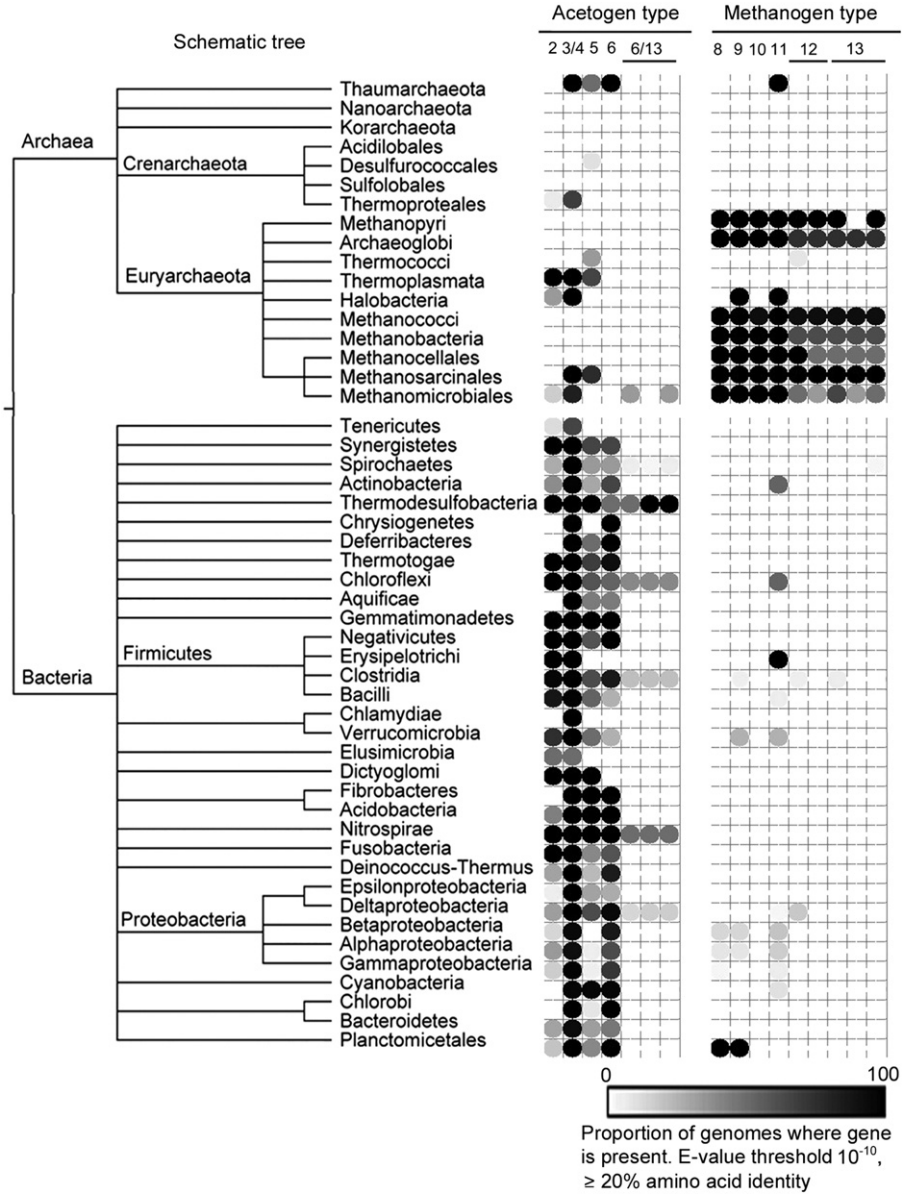


Fig. 5. Distribution of the genes from the WL-pathway. The left part of the figure represents the organization of the selected taxonomic groups from 1606 completed sequenced genomes (117 archaeal and 1489 eubacterial). The right part of the figure represents the proportion of genomes within a taxon where the gene is present. Each column represents a different gene and numbers correspond to the reactions presented in Table 1. Homologous proteins involved in the several steps of the acetogen type (2: *Fhs*, 3/4: *folD*, 5: *metF*, 6: *ascE*/*MeTr*, 13: *cdh* δ/*acsD*, *cdh* γ/*acsC*, and *cdh* (α/β)/*acsB*) and methanogen type (8: *Ftr*, 9: *mch*, 10: *mtd*, 11: *mer*, 12: *cdh* γ/*acsC* and *cdh* δ/*acsD*, 13: *cdh* α_{1-4}, *cdh* ε_{1-2} and cdhβ) were identifyed by BLAST (E value threshold of 10^{-10} and amino acid identities of at least 20%).

been suspected to be a site of energetic coupling [226], via a mechanism that might involve electron bifurcation [146]. Thus, the *Moorella* and *Methanothermobacter* methylene-pterin reductase enzymes are related, but the methanogen enzyme is structurally more similar to the luciferase superfamily than it is to MetF and there exists some diversity among bacterial methylene-H_4F reductases as well [85].

Reactions 6 and 12 entail the methyltransferase that catalyzes the transfer of the pterin-bound methyl group to cobamid in CoFeS. *Moorella* MeTr (PDB ID: 4djd chain A, B) consists of a TIM-barrel [225] and belongs to the dihydropteroate synthetase-like (tethyltetrahydrofolate — utilizing methyltransferases) SCOP superfamily. Because of translational fusions, *Moorella* MeTr links steps 6 and 12 to CODH/ACS, reaction 13 (Fig. 4e). There is considerable structural information for the bacterial enzymes, including *Moorella* ([43,225]). There are no structures of the archaeal AcsD subunit, but in sequence comparisons at SUPERFAMILY [65] it returns the same SCOP superfamily as *Moorella* MeTr and it is well known that the archeaeal and bacterial CODH/ACS are homologous [156,175], although Cdhε from *M. barker*i (PDB ID: 3CF4 chain G; [64]) does not have a homologue in the *Moorella* enzyme. For Cdhα from *M. barkeri* (PDB ID: 3CF4 chain A) the structure belongs to the acetyl-CoA synthase SCOP superfamily [64]. These corrinoid-dependent methyltransferases, and methionine synthase (MetH) all share a common TIM-barrel fold and are related [14,155]. They are furthermore related to the methyltransferase system of CoFeSP [225].

Thus, through structural comparison using the DALI server [84] we found common folds among some enzymes from the methyl synthesis branch of the acetyl CoA pathway in acetogens and methanogens (Fig. 4) where amino acid similarity was lacking (Table 1), in particular we found shared TIM barrel containing protein families. Even in the case of the methylene-pterin dehydrogenases (Fig. 4c), DALI alignments find higher similarity scores for the proteins to other families. Structural evolutionary studies of the TIM barrel fold have delivered arguments both in favor of divergent [53] and convergent evolution [102] of this domain. Moreover, even studies supporting divergent evolution do not reach a consensus of which group(s) of TIM barrel containing protein families shared a common ancestor ([6,31,130,131,158,213,227]). Thus, these weak structural similarities are consistent with the view that the two methyl-synthesis branches of the Wood–Ljungdahl pathway evolved independently in the ancestors of *Moorella* and *Methanothermobacter* (Fig. 4). Moreover, because of the patterns of sequence conservation that we observe for the acetogen and methanogen versions of the WL among prokaryotes more generally (Fig. 5) the conclusion can be extended that, these weak structural similarities are consistent with the view that the two methyl-synthesis branches of the Wood–Ljungdahl pathway evolved independently in the ancestors of acetogens and methanogens, even though there are some common domains among enzymes. The exceptions are the corrinoid dependent methyltransferases, which are all interrelated [155].

In the acetogen–methanogen comparison of the acetyl-CoA pathway, the acetyl synthesis segment and the methyltransferase system that donates methyl groups to CODH/ACS are clearly homologous and, as previously proposed ([185,225]), evolutionarily related. The remaining enzymes of methyl synthesis segment are not related (except for a few shared domains). The methyl-pteridine:cobamide methyltransferases and corresponding domains in CoFeS and CODH/ACS belong to the same SCOP superfamily. Svetlitchnaia et al. [185] have suggested that the more complicated methionine synthase family arose from the methyltransferase system of CoFeSP in the acetyl-CoA pathway, which would further point to the antiquity of this CO_2-fixation route.

3.6. Discriminating between some alternative theories

The present findings permit discrimination between some competing alternatives for the evolution of the WL-pathway (Fig. 6). Braakman and Smith [22,23] suggested that the acetyl-CoA pathway was present in the last common ancestor of all cells ('Luca', which we take to mean the last common ancestor of prokaryotes), with subsequent vertical inheritance of the pathway into the bacterial and archaeal lineages, which generates the expected patterns of homology that are shown in Fig. 6a. Nitschke and Russell [133] suggested that a denitrifying methanotrophic WL-pathway was present in 'Luca' and that methanogenesis arose late and altogether independently from acetogenesis, which generates the expected patterns of homology that are shown in Fig. 6b. Ferry and House [56] suggested that the acetyl synthesis segment of the WL pathway was present in the last common ancestor, as did Martin and Russell [115] who, like Sousa et al. [179], furthermore suggested that the methyl synthesis segments arose independently in the acetogen and methanogen lineages. That view generates the expected patterns of homology that are shown in Fig. 6c. The observation from sequence (Table 1) and structural comparisons (Fig. 4) is summarized in Fig. 6d.

The recent investigations of Braakman and Smith [22,23] clearly predict that the enzymes of methyl synthesis in the WL-pathway should be homologous and related in acetogen-methanogen comparisons, which is however not the case. How can that be? Their "phylometabolic" method infers gene presence or absence based upon sequence annotation data rather than being based upon sequence similarity or structure similarity data. It was likely the reliance upon database annotations that led them to infer the presence of a complete WL-pathway in 'Luca' [22,23], a conclusion that is, however, incompatible with both earlier studies [109] and with the sequence, structure and cofactor comparisons (Table 1, Figs. 3, 4) reported here.

The suggestion of Nitschke and Russell [133] that the acetyl-CoA pathway and in particular its manifestation in methanogenesis is not ancient has several problems. The schematic phylogenetic tree in their Fig. 2 that depicts methanogens arising very late in evolution is incompatible with current studies of deep phylogeny (reviewed by [215]). Furthermore, and as pointed out previously [179], the denitrifying methanotrophy model requires oxidizing conditions (that is, high concentrations of NO and/or nitrite) in the ocean; but under even the most mildly oxidizing conditions, the accumulation of reduced organic compounds at the vent ocean interface is no longer thermodynamically favorable [119]. Finally, the biological process of methanogenesis has an observable homologue at hydrothermal vents in the form of continuous geochemical synthesis of methane and other organic compounds in serpentinizing systems [52,100,151]. By contrast, even under today's oxic atmosphere, geochemical methane oxidation, which the model of Nitschke and Russell [133] requires, has so far not been reported, likely owing to the very high bond energy of the C–H bond in methane [168], suggesting that denitrifying methanotrophy is a product of biological evolution, not the source of its origin.

In summary, the current data from sequence and structural comparisons are most compatible with a scenario in which acetyl synthesis was present in 'Luca', while the methyl synthesis segment arose later and independently in the stem lineages leading to acetogens and methanogens (Fig. 6d). That observation is furthermore consistent with the distribution of genes for H_4F and H_4MPT cofactor biosynthesis in Fig. 2.

3.7. Demands on spontaneous synthesis — asking for miracles?

As Ferry and House [56] have suggested, and as we have also suggested but for different reasons [115,179], substrate level energy conservation entailing thioester synthesis and acyl phosphate synthesis as it occurs in acetogens or some methanogens grown on CO [161] could be a sustained source of harnesable chemical energy in chemical and early biological evolution. But that, in turn, requires a sustained source of geochemical methyl groups before and during of genes and proteins [114]. How might such

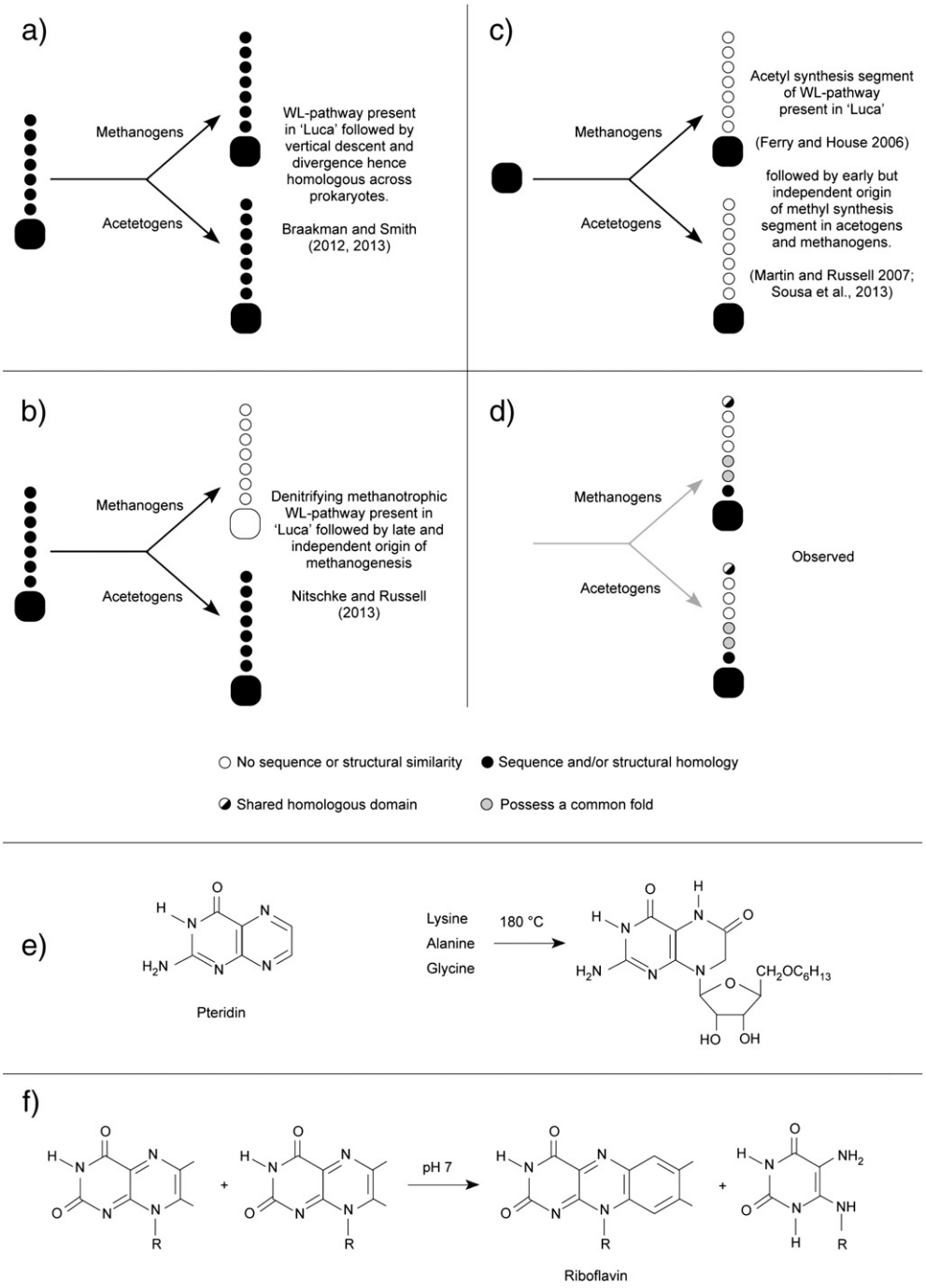


Fig. 6. Looking back into time along the WL-pathway. a–c: Alternative views on the origin of the Wood–Ljungdahl (WL) pathway and abiotic pterin reactions. The reactions from the methyl segment (reactions 1 to 6 and 7 to 12) are represented by small circles and the ones from the acetyl-CoA segment by a large square (reaction 13). Open circles represent absence of sequence and structural similarity, half-colored circles indicate the sharing of homologous domains, black circles indicate sequence and/or structural similarity and gray circles the presence of a common fold. a. WL-present in "Luca" followed by vertical descent and divergence hence homologous across prokaryotic domains [22,23]. b. Denitrifying methanotrophic WL-pathway present in "Luca" followed by late and independent origin of methanogenesis [133]. c. Acetyl synthesis segment of WL-pathway present in "Luca" [56] followed by early but independent origin of the methyl synthesis segment in acetogens and methanogens [115,179]. d. Observed distribution of homologies within the pathway according to structural and sequence comparisons in this paper. e. A pterin structure and abiotic formation of a pterin from amino acids (compound 7 in [80]) f. Synthesis of riboflavin either via riboflavin synthase [57] or spontaneously without enzymes at pH 7 [138].

compounds have been formed? McCollom et al. [120] have shown that the gas–water shift reaction:

$$CO_2 + H_2 \leftrightarrow CO + H_2O \quad (1)$$

is a ready source of reactive C1 compounds (CO and formate) under simulated high-temperature (ca. 250 °C) hydrothermal vent conditions in the laboratory both with and without added iron. Over periods of hours to days, several percent of the carbon is reduced to Fischer–Tropsch type reaction products [122]. Also relevant in this context is a new enzyme of CO_2 reduction in *A. woodii* that has recently been discovered, a hydrogen dependent carbon dioxide reductase that catalyzes the reduction of CO_2 with H_2 to formic acid. The enzyme is Mo-dependent and otherwise has no organic cofactors, only FeS clusters [140,170]. And Lost City today generates formate, at least in micromolar amounts [100], and methane — generated from CO_2 via geochemical carbon

reduction processes [121] — in millimolar amounts [151]; methane is also observed in terrestrial serpentinizing systems [52]. Thus the inference that the progenote arose and existed in an environment that was continuously supplied with chemically accessible C1 compounds is not fundamentally problematic (that is, it is compatible with present observations). Regarding methyl groups specifically, Loison et al. [108] used simulated hydrothermal conditions to react CO (ca. 40 μM) and H_2S in the presence of Ni^{2+} at 90 °C for hours to days. They obtained mixtures of de novo synthesized products replete with methyl groups including methylsulfide, dimethylsulfide, methylethylsulfide, formate, acetate, propionate and many short branched-chain compounds, the latter indicating that the synthesis was not a standard Fischer–Tropsch type reaction. Reactions of these types serve to exemplify the kind of geochemical synthesis of reactive C1 compounds that we have in mind.

The synthesis of formyl pterins from CO_2 requires an uphill energy investment (Fig. 3). But the energetics of the acetyl-CoA pathway are such that the energy rich thioester can, in principle, be synthesized continuously, provided that there is an environmental source of methyl groups, for example methyl sulfide [79,86], although abiotic methyl sulfide has not been reported from hydrothermal vents so far. Though not shown in Fig. 3, energy conservation in both methanogens and acetogens requires chemiosmotic ion pumping, because there is not enough energy in the pathway starting from H_2 and CO_2 to support carbon and energy metabolism via substrate level phosphorylation alone [55,61,115,128,190]. Also not shown in Fig. 3, the operation of the pathway in both acetogens and methanogens depends upon flavin-dependent electron bifurcation [26] in order to generate low potential ferredoxins with electrons from H_2.

And what about the abiotic synthesis of pterins themselves? Sutherland and Whitfield [184] suggested a synthesis based on HCN. However, Heinz et al. [80] heated equimolar amounts of lysine, alanine, and glycine at 180 °C for several hours and obtained various pterins, one of which is shown in Fig. 6e. In addition to pterins, they also obtained several flavins. This type of synthesis has been repeated very often with similar results [189], and while one might complain that those conditions have little to do with alkaline hydrothermal vents, the observation raises a point worth mentioning, namely that enzymes do not create new reactions, they optimize existing ones [50]. For example, the reaction in Fig. 6f shows the biosynthesis of riboflavin, a rather complicated reaction catalyzed by the enzyme riboflavin synthase [57]. However, the reaction also occurs spontaneously at neutral pH without the help of enzymes [138]. That is of interest when one discusses flavin-based electron bifurcation as a possibly very ancient mechanism in early biochemistry [82]. It might seem virtually impossible at first sight that such complicated cofactors as flavins could arise without immense catalytic help. But such spontaneous, non-enzymatic flavin-generating reactions suggest that such compounds are more "natural" than one might think. Perhaps cofactor structures have something genuinely "predisposed" about them [184]. Even a cofactor as complicated as vitamin B_{12} can have many structural components that seem to spontaneously fall into place. Eschenmoser [51] wrote about B_{12}: "*…the A/D-ring junction, regarded as the main obstacle to a chemical vitamin B_{12} synthesis at the outset, is in fact a structural element that is formed readily and in a variety of ways from structurally appropriate precursors […] the same holds for other specific structural elements of the vitamin B_{12} molecule, including the characteristic arrangement of double bonds in the corrin chromophore, the special dimension of the macrocyclic ring of the corrin ligand, the specific attachment of the nucleotide loop to the propionic acid side chain of ring D, and the characteristic constitutional arrangement of the side chains around the ligand periphery (which vitamin B_{12} shares with all uroporphinoid cofactors). All these outwardly complex structural elements are found to 'self-assemble' with surprising ease under structurally appropriate preconditions; the amount of 'external instruction' required for their formation turns out to be surprisingly small in view of the complexity and specificity of these structural elements.*"

3.8. A role for geochemical methyl groups in translation

In modern hydrothermal systems, methane of abiogenic origin is common [52,151]. The reducing power to convert CO_2 into methane comes from the process of serpentinization [121], a series of geochemical reactions in which Fe^{2+} in the submarine crust reduces H_2O in hydrothermal systems to H_2 and inorganic carbon to abiogenic CH_4, which can be present in amounts up to 10 mM in vent effluent [151]. Serpentinization has been occurring since there was liquid water on Earth [8,177]. Although methyl compounds of abiogenic origin have not been reported in hydrothermal effluent so far (Tom McCollom, personal communication), methane synthesis in serpentinizing systems is a rather sure indicator that methyl groups are being generated as intermediates in hydrothermal systems [121,169]. Accordingly, our suggestion for the source of methyl groups to feed acetyl thioester synthesis during the phase of evolution prior to the origin of either methyl synthesis branch of the Wood–Ljungdahl pathway is that these were spontaneously synthesized geochemically in serpentinizing hydrothermal systems. Are some methyl groups relicts from the ancient past?

The ribosome of both archaea and bacteria is methylated, particularly around the peptidyl transferase site [75]. In addition to the ribosome, most tRNAs contain modified bases, and a number of those modified bases are shared by bacteria and archaea [28,143]. These modifications include the introduction of sulfur atoms, thiomethyl groups, acetyl groups isoprene groups and the like [143], but by far the most common modifications both of the ribosomal RNA and tRNA bases are methylations. One view has it that RNA methylation is a comparatively late appearance in chemical evolution [59] and might represent a kind of intermediate state in the transition from RNA to DNA as the genetic material [147]. The prevalence and universal conservation of some sulfur atoms, thoimethyl groups, acetyl groups and many methyl groups in RNA modifications would not fit very well in that view. An alternative view has it that RNA methylations and base modifications are holdovers from the chemical environment where the RNA world, the genetic code, the progenote and life arose — a chemically reactive and far from equilibrium environment rich in sulfur and replete with chemically reactive methyl groups [114,179]. In that view, the modern enzymatic introduction of such RNA base modifications would be a means to recreate the ancestral state. In other words, conserved methylations in ribosome-tRNA interactions might be a window into the workings of the ribosome at a time when bases were synthesized spontaneously, without extensive help from genes.

In metabolism, the methyl groups that are introduced into rRNA and tRNA come from S-adenosyl methionine, SAM [13,27,221] and are transferred to the 2′ OH of ribose or the bases themselves by base modifying enzymes, many of which belong to the radical SAM family [13,27,145]. Even the synthesis of other methyl-carriers such as methanopterins, require radical SAM enzymes [209]. Radical SAM enzymes have FeS clusters and generate a radical intermediate in the reaction mechanism [71]. FeS clusters and radical enzyme mechanisms are likely ancient biochemical traits [72,96] and their involvement would be consistent with the general antiquity of base methylations in rRNA-tRNA interactions. The five amino acid methylations in the active site of methyl-CoM reductase [48] might also be seen as similar relicts from environmental chemistry.

3.9. Archaea: ancestrally methanogenic?

The ubiquity of H_4MPT biosynthesis genes among the archaea, allowing for lateral acquisition of H_4F in haloarchaea, suggests that H_4MPT was the C1 carrier in the archaeal common ancestor. But was the archaeal common ancestor a methanogen?

Methanogens are clearly ancient [105], biogenic methane is also clearly ancient, going back some 3.5 Ga [193], some phylogenetic analyses implicate methanogens as the ancestral archaeal group [92,215], methanogens are replete with metal and metal sulfide catalysts

[110] in the proteins they use in the acetyl-CoA pathway, which is also clearly ancient [16,61], serpentinization generates methane abiotically from H_2 and CO_2 [121] and serpentinization is clearly ancient [8], methanogens use H_2S instead of cysteine as precursor of iron–sulfur centers [106], and contain 21 times more acid labile sulfide than *E. coli* cells [211] which can be seen as a relict of their ancient metabolism. But similar arguments could be generated for sulfate reducers with regard to the 3.5 Ga age of the process [142], the use of the acetyl CoA pathway in autotrophic sulfate reducers [153], the frequency of metal and metal sulfide catalysts in their proteins [179], and the environmental availability of the energy metabolic substrates H_2 and SO_2 [8] — the exergonic reactions in sulfate reduction start with sulfite [190] which ensues from SO_2 contact with water.

The involvement of cytochromes in sulfate reducers prompted Decker et al. [37] to suggest that they are a more recent arrival on the evolutionary stage than methanogens that lack cytochromes, and that argument still seems robust [179]. Also, carbon and energy metabolism in methanogens both involve the acetyl-CoA pathway, but these are separated in sulfate reducers into distinct carbon and energy metabolic routes [139,153], whereby many sulfate reducers use the acetyl-CoA pathway. Separation would allow carbon and energy metabolism to evolve independently in sulfate reducers, providing freedom for diversity in energy metabolic routes [167] including quinone-dependent ion pumping, while the methanogens remained more specialized due to the nature of their coupled carbon and energy metabolism.

When the theory of pyrite energetics was first presented, Wächtershäuser [196] mentioned methanogenesis and sulfur reduction with electrons from H_2 as possible primordial energy sources for the archaea, before discarding both in favor of pyrite formation. Methanogenesis was discarded because it required an 'energized coupler' to link exergonic steps in methane generation to the endergonic reduction of CO_2 and the energized coupler was seen as a product of evolution, hence derived, not ancient [196]. Twenty five years later, flavin based electron bifurcation is found to mediate the corresponding steps of methanogenesis, and the 'energized coupler' turns out to be FeS clusters in reduced ferredoxin [26,89]. Thus, methanogenesis is a good candidate for ancestral archaeal energy metabolism once again, and as Herrmann et al. [82] point out, reduced ferredoxin has the attributes of an energy currency more primitive than ATP. Furthermore, what once seemed to be an insurmountable leap in complexity, the origin of chemiosmotic coupling — an aspect that Wächtershäuser never integrated well into the otherwise robust FeS theory — can now be readily understood in the context of naturally preexisting proton gradients at alkaline hydrothermal vents [163,164]. Those natural gradients could be harnessed with the advent of genes and proteins, leaving the hardest step for last: the invention of machines to replace the prexisting ion gradient by coupling exergonic reactions to ion pumping [99], and thus become energetically independent from the geochemical ion gradient at the vent. Good candidates for such ancestral pumping systems are the MtrA–H complex of cytochrome-lacking methanogens, which pumps sodium while transferring the methyl moiety from methyl H_4MPT to CoM [191] and the Rnf complex in cytochrome-lacking acetogens that pumps sodium while reducing NAD^+ with electrons from reduced ferredoxin [18]. In both groups, the synthesis of low potential reduced ferredoxin is dependent upon electron bifurcation [26].

In that context, one could consider the sole coupling reaction of methanogens that lack cytochromes — the exergonic transfer of a methyl group from a nitrogen atom to a sulfur atom by the MtrA–H methyltransferase complex — in a more ancient light. At the fringes of a hydrothermal vent where natural ion gradients, harnessable by the universal ATPase, were dissipating, but geochemically generated methyl moieties were still abundant, such a "substrate level pumping" (methyltransferase) reaction might have required a lower level of evolutionary invention than the coupling of ion pumping to electron transfers. Might the methyltransferase reaction be the most ancient ion pumping step in the archaeal lineage? It would fit well with the notion that life arose in an environment rich in geochemical methyl groups and with the idea that methanogenesis is the earliest form of chemiosmotic energy conservation in archaea.

The deep dichotomy of archaea and bacteria is evident at the level of ribosomal protein composition [198], membrane lipid synthesis [94], cell wall constituents [2], and flagellar composition [87] in addition to other traits. That deep dichotomy is further underscored by the distribution of genes for the synthesis of H_4F in bacteria and H_4MPT in archaea, respectively. Furthermore, the enzymes underlying those distinct biosynthetic routes are broadly unrelated across the bacterial–archaeal divide (Fig. 2), indicating that the corresponding pathways arose independently subsequent to divergence from a progenote-like last universal common ancestor, which from the standpoint of gene content appears to have been more or less a geochemically fuelled ribosome-and-code complex. That deep divergence is also mirrored in the different enzymes and cofactors that archaea and bacteria use to perform methyl synthesis for methanogenesis and acetogenesis respectively (Fig. 3, Table 1), while acetyl synthesis at CODH/ACS is conserved among acetogens and methanogens and involves transition metal catalysis. Thus, the acetyl-CoA pathway, while being the most ancient of known CO_2 assimilation pathways [61], reflects two phases in early evolution: an ancient one in the progenote, in which acetyl thioester synthesis proceeded with the help of spontaneously (geochemically) supplied methyl groups, and a later phase that reflects the primordial divergence of the bacterial and archaeal stem groups and their independent inventions of genetically-encoded means to synthesize methyl groups via enzymatic pathways.

4. Conclusions

Of course, the acetyl-CoA pathway is not universal, it was superceded by other routes of CO_2 assimilation and energy metabolism that were invented and inherited (both laterally and vertically) during microbial evolution [61,192]. But it appears to be the evolutionary starting point of carbon and energy metabolism in both archaea and bacteria — reflecting a chemistry that i) is more ancient than the genes that catalyze it today, and that ii) has a living fossil 'sister group' in the form of geochemical methane synthesis at modern hydrothermal vents.

The ubiquity of H_4MPT biosynthesis genes among archaea is compatible with the view that the first free-living members of the archaeal domain were methanogens. However, new and potentially more ancient kinds of energy metabolism involving sulfur are still being discovered [123]. Conserved acetyl thioester synthesis in the acetyl-CoA pathway, together with independently invented methyl synthesis pathways using independently invented pterin C1 carriers, appear to hold clues about the energetic and chemical environment within which the progenote and its descendant stem lineages arose. The prevalence of methyl groups in the chemically modified bases in the ribosome and tRNAs also, in our view, points to the environment in which the progenote navigated the transition from geoenergetics and geosynthesis to bioenergetics and biosynthesis, within the confines of naturally forming inorganic microcompartments at a Hadean hydrothermal vent, one that was rich in reactive methyl groups, a world of one carbon compounds, or a 'C1 world', were one so inclined.

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Appendix A. Supplementary data

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