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Diversity and Evolution of Methane-Related Pathways in Archaea

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Abstract

Methane is one of the most important greenhouse gases on Earth and holds an important place in the global carbon cycle. Archaea are the only organisms that use methanogenesis to produce energy and rely on the methyl–coenzyme M reductase complex (Mcr). Over the last decade, new results have significantly reshaped our view of the diversity of methane-related pathways in the Archaea. Many new lineages that synthesize or use methane have been identified across the whole archaeal tree, leading to a greatly expanded diversity of substrates and mechanisms. In this review, we present the state of the art of these advances and how they challenge established scenarios of the origin and evolution of methanogenesis, and we discuss the potential trajectories that may have led to this strikingly wide range of metabolisms.

Contents

1. INTRODUCTION

Methane is one of the major greenhouse gases and is crucial to current global environmental changes (90). Biological production of methane may have started as early as 3.5 Ga (107), with a direct impact on the evolution of the Earth's climate (49). Archaea are the only organisms that generate methane as a by-product of their energy metabolism. Methanogenic archaea (or methanogens) are strict anaerobes dwelling in all types of anoxic niches, from sediments and rice paddies to the gastrointestinal tracts of animals (64, 104). In most of these environments, they form syntrophic partnerships with fermentative bacteria (93), allowing the complete degradation of organic matter and therefore acting as major players in the global carbon cycle (26) (**Supplemental Figure 1**). It is estimated that through these associations, methanogens are responsible for up to 50% of organic matter mineralization in freshwater environments (9) and for the overall production of 1 Gt of methane per year (100). Only half of this methane reaches the atmosphere (25, 88), because it is consumed by anaerobic archaeal methanotrophs as well as anaerobic and aerobic bacterial methanotrophs.

Supplemental Material >

Most known methanogens are highly specialized microorganisms that can only use a narrow range of one- or two-carbon compounds to obtain energy (**Figure 1*a*,*b***). A key enzymatic complex of both archaeal methanogens and methanotrophs is the methyl–coenzyme M (CoM) reductase complex (Mcr), which is directly responsible for the production and/or oxidation of methane (101). Methanogenesis pathways have been historically grouped into three categories: (*a*) hydrogenotrophic, for the reduction of CO_2 into CH_4 using H_2 as the electron donor, (*b*) methylotrophic, for the dismutation of methyl compounds into CH_4 and CO_2, and (*c*) acetoclastic, for the dismutation of acetate into CH_4 and CO_2. The reduction of methyl compounds with H_2 has often been classified as methylotrophic. However, since hydrogen is the electron donor in this reaction, it would be more correct to qualify it as hydrogenotrophic. We thus propose referring to this pathway as "methyl-reducing hydrogenotrophic methanogenesis" and, to

avoid confusion, using "CO_2-reducing hydrogenotrophic methanogenesis" for the reduction of CO_2 into CH_4 using H_2 as an electron donor (**Figure 1*a***). More broadly, the terms CO_2-reducing methanogenesis and methyl-reducing methanogenesis may also be used to include cases where alcohol or formate is the electron donor. In this review we also use the term methyl-dismutating methanogenesis instead of methylotrophic methanogenesis when methyl groups are used as both carbon and electron donors for methanogenesis (**Figure 1*a***).

Studies on methanogenesis and methanogens started in the late nineteenth century (94), and over 200 species capable of this metabolism are currently characterized. Until 2012, all known methanogens were classified into six orders, gathered into Class I, comprising *Methanobacteriales*, *Methanococcales*, and *Methanopyrales*, and Class II, comprising *Methanosarcinales*, *Methanomicrobiales*, and *Methanocellales* (7) (**Figure 1*c***). These archaea mostly perform CO_2-reducing methanogenesis and use the methyl branch of the Wood-Ljungdahl pathway (MBWL), as well as Mtr (tetrahydromethanopterin *S*-methyl-transferase complex) and Mcr (92) (**Figure 1*b***). These enzymes are conserved in all Class I and Class II methanogens, with the exception of *Methanimicrococcus* (105), regardless of their methane metabolism (**Figure 1*c***).

In only a decade, metagenomic approaches have revealed a wealth of novel deep-branching lineages of Mcr-containing archaea (**Figure 1*c***). Strikingly, of these newly discovered lineages, only two ["*Candidatus* Methanohydrogenales"/"*Ca.* Nezhaarchaeota" (11, 112) and "*Ca.* Methanomixophus" in *Archaeoglobales* (63, 112)] are predicted to be CO_2-reducing methanogens, while the vast majority are methyl-reducing hydrogenotrophic methanogens. The latter belong to the *Methanotecta* [*Methanonatronarchaeia* (96)], the *Diaforarchaea* [*Methanomassiliicoccales* (16, 30)], the *Acherontia* ["*Ca.* Nuwarchaeales" (NM3) (15, 114), "*Ca.* Methanofastidiosa" (81)], the TACK ["*Ca.* Methanomethyliales" (108), "*Ca.* Methanodesulfokores" (15, 72)], and a basal *Thaumarchaeota* lineage (47). These methanogens rely on substrate-specific methyltransferases, and all lack Mtr and a complete MBWL pathway (15) (**Figure 1*a***). Methyl-reducing hydrogenotrophic methanogenesis, previously thought to have a very narrow distribution, is now known to be the most widely distributed across all the Archaea phylogeny.

All reactions taking place in the core enzymes of CO_2-reducing methanogenesis are fully reversible, allowing the anaerobic oxidation of CH_4 into CO_2. The possibility that such capacity exists in Archaea was first suggested in the 1990s (46) and was later supported by microscopic (13), isotopic (83), and metagenomic evidence (42). Anaerobic methanotrophs (ANME) belong to "*Ca.* Methanophagales" (ANME-1) and several lineages in the *Methanosarcinales* (53) (**Figure 1*c***). Several names have been proposed for ANME lineages in *Methanosarcinales*: ANME-2d "*Ca.* Methanoperedens" (43), ANME-2a "*Ca.* Methanocomedens," ANME-2b "*Ca.* Methanomarinus," ANME-2c "*Ca.* Methanogasteraceae," and ANME-3 "*Ca.* Methanovorans" (21). Marine ANME archaea transfer electrons from the oxidation of methane to syntrophic sulfate-reducing bacteria (SRB) affiliated with the *Desulfobacterales* (53), while freshwater lineages can directly reduce inorganic compounds such as Fe(III) (61). The recent availability of genomic data from members of all identified ANME clades has now enabled more detailed predictions on the functioning of anaerobic methanotrophy (21, 106). It was also suggested that "*Ca.* Methanomixophus" (63) and "*Ca.* Methanodesulfokores" might be capable of this metabolism (72).

Another recent exceptional finding brought by exploration of the uncultured diversity of Mcr-containing archaea was the discovery of many lineages predicted to use multicarbon alkanes ranging from 2 carbons (ethane) to more than 39 carbons as a source of energy (113, 124) (**Figure 1*b***). This metabolism, termed alkanotrophy, was first proposed in the "*Ca.* Syntropharchaeales," which oxidize short-chain alkanes [propane (C_3) and butane (C_4)] into CO_2 by using an alkyl-CoM reductase (Acr), a distant homolog of Mcr, combined with the β-oxidation and Wood-Ljungdahl pathways (58). Additional Mcr-like homologs, likely also involved in the activation of

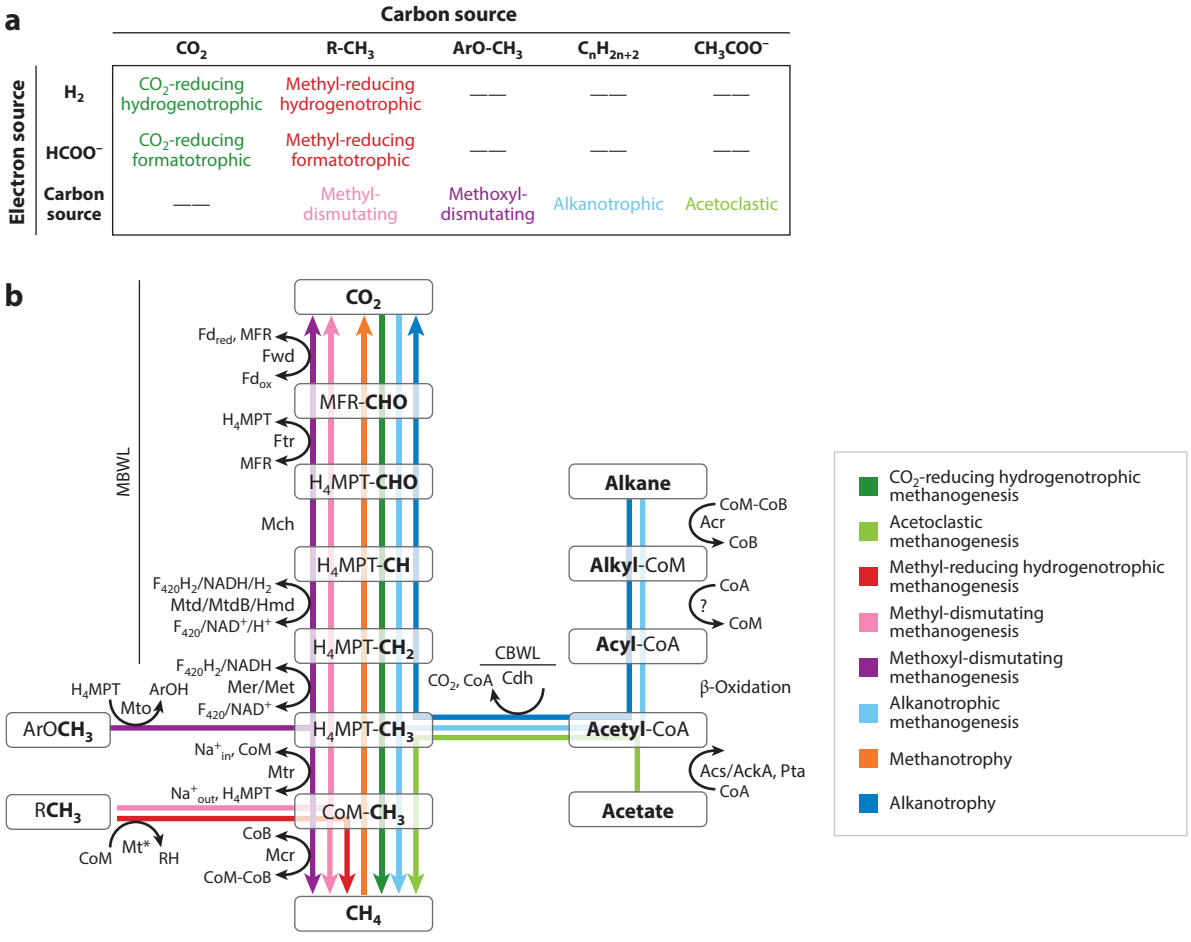

Figure 1

(*a*) Nomenclature of different types of methanogenesis, depending on the carbon and electron sources. (*b*) Currently known methane-related pathways. Major metabolites are boxed, and carbon groups are indicated in bold. The asterisk with Mt indicates different methyltransferases (**Supplemental Tables 1 and 2**). (*c*) Taxonomic distribution of methane-related pathways across the reference phylogeny of Archaea. Colored squares at the tips of branches correspond to the presence of each type of metabolism. The proportions of genomes of each taxon coding for main components of the pathways are indicated by color gradient (0%, 1–25%, 26–50%, 51–75%, 76–100%). The different colors correspond to functional categories. The analysis was conducted on 832 archaeal genomes, and the presence of complexes or pathways was determined using MacSyFinder (1) (**Supplemental Table 3**). Because MHC is part of a large protein family, the average number of homologs per genome (identified by proteins containing at least two CxxCH motifs) is indicated (minimum, 0.0; maximum, 17.0). For expanded names of all abbreviated enzymes, see the sidebar titled Terms and Definitions. Abbreviations: Acr, alkyl-CoM reductase complex; ANME, anaerobic methanotrophs; CBWL, carbonyl branch of the Wood-Ljungdahl pathway; CoA, coenzyme A; DPANN, "*Ca*. Diapherotrites," "*Ca*. Parvarchaeota," "*Ca*. Aenigmarchaeota," "*Ca*. Nanohaloarchaeota," and "*Ca*. Nanoarchaeota"; Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin; H_4MPT, tetrahydromethanopterin; MBWL, methyl branch of the Wood-Ljungdahl pathway; Mcr, methyl-coenzyme M reductase complex; MFR, methanofuran; MHC, multi-heme cytochrome; Mtr, tetrahydromethanopterin *S*-methyl-transferase complex; TACK, superphylum composed notably of *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and "*Ca*. Korarchaeota."

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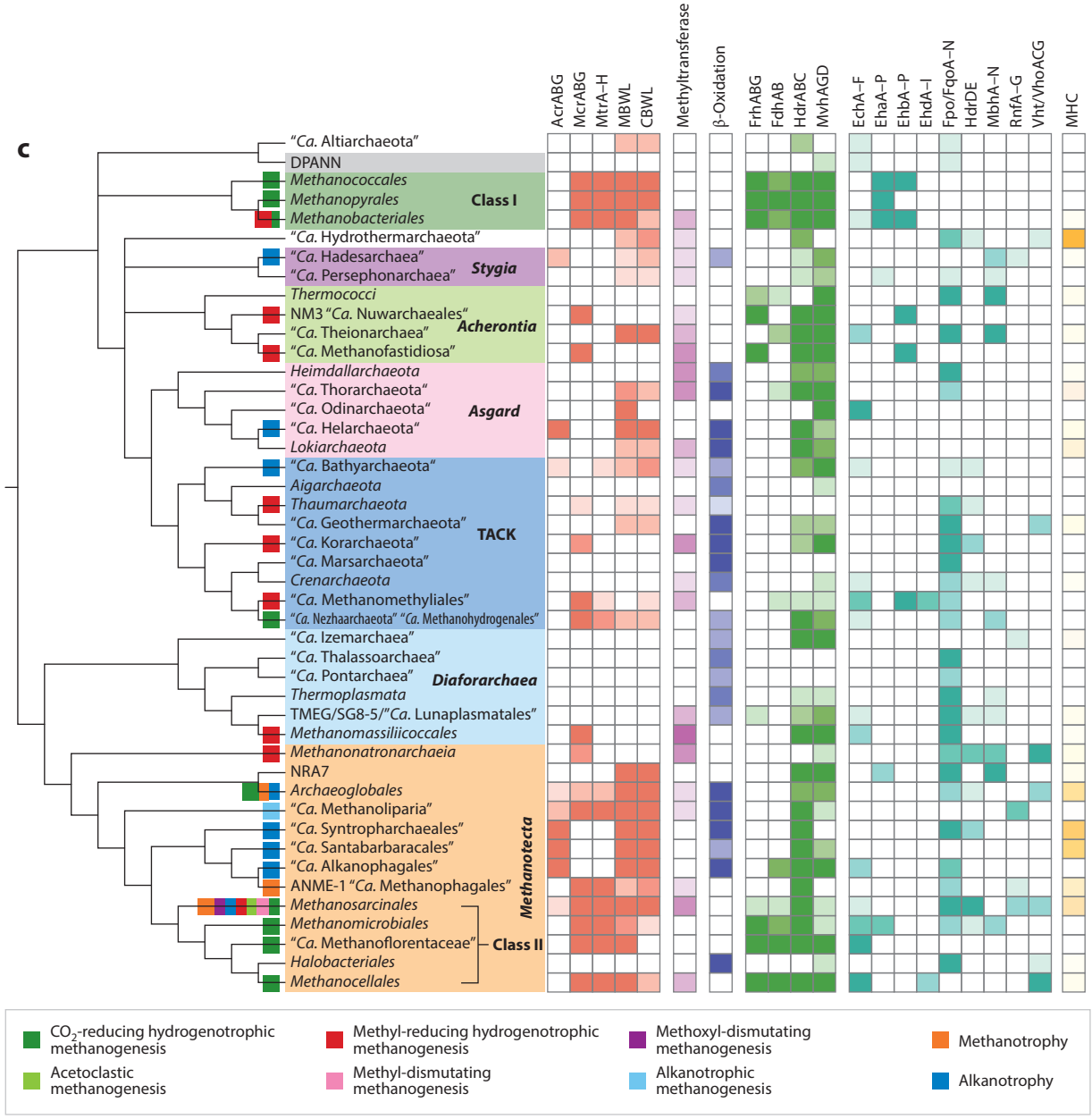

Figure 1

(*Continued*)

multicarbon alkanes, were found in several members of the *Stygia* ["*Ca*. Hadesarchaea" (47, 112)], *Asgard* ["*Ca*. Helarchaeota" (91, 122)], TACK ["*Ca*. Bathyarchaeota" (34)], and *Methanotecta* ["*Ca*. Methanoliparia" (15, 57, 124), *Archaeoglobales* (18), "*Ca*. Santabarbaracales" (114), "*Ca*. Alkanophagales" (114), *Methanosarcinales* (15, 22, 40)]. Most of these lineages have the same core enzymes as

"*Ca*. Syntropharchaeales" (**Figure 1*c***). However, several enzymatic steps between the initial activation of the alkyl and the β-oxidation pathway are still unknown (113).

TERMS AND DEFINITIONS

The following abbreviations of taxa, enzymes, and cofactors are used throughout this article, in the main text and figures.

TAXA/ORGANISMS

ANME: anaerobic methanotrophs
DPANN: superphylum composed notably of "*Ca*. Diapherotrites," "*Ca*. Parvarchaeota," "*Ca*. Aenigmarchaeota," "*Ca*. Nanohaloarchaeota," and "*Ca*. Nanoarchaeota"
SRB: sulfate-reducing bacteria
TACK: superphylum composed notably of *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and "*Ca*. Korarchaeota"

CENTRAL PATHWAY

Ack: acetate kinase
Acr: alkyl–coenzyme M reductase complex. Involved in alkane (other than methane) oxidation
Acs: acetyl-CoA synthase
CBWL: carbonyl branch of the Wood-Ljungdahl pathway (Cdh)
Cdh: CO_2 dehydrogenase complex
Ecr: ethyl–coenzyme M reductase complex. A subtype of Acr specialized in ethane utilization
Ftr: formyl-methanofuran-tetrahydromethanopterin formyl-transferase
Fwd: formyl-methanofuran dehydrogenase complex
Hmd: methylene-tetrahydromethanopterin dehydrogenase, H_2 dependent
Lcr: long-chain alkyl–coenzyme M reductase complex. A subtype of Acr specialized in long-chain alkane utilization (e.g., hexadecane)
MBWL: methyl branch of the Wood-Ljungdahl pathway. In methanogenic and methanotrophic archaea, it is composed of Fwd, Ftr, Mch, Mtd/MtdB/Hmd, and Mer/Met and uses the H_4MPT cofactor
Mch: methenyl-tetrahydromethanopterin cyclohydrolase
Mcr: methyl–coenzyme M reductase complex. Involved in methyl reduction during methanogenesis and methane oxidation during methanotrophy
Mer: methylene-tetrahydromethanopterin reductase
Met: methylene-tetrahydrofolate reductase complex (likely a methylene-tetrahydromethanopterin reductase in alkanotrophic archaea)
MT1: methyl-substrate:corinoid protein methyltransferases (correspond to MtaB, MtmB, MtbB, MttB, MtoB, MtpA, MtgB)
MT2: corinoid protein:coenzyme M methyltransferases (correspond to MtaA, MtbA, MtoA, MtsF, MtgA)
Mtd: methylene-tetrahydromethanopterin dehydrogenase, F_{420} dependent
MtdB: methylene-tetrahydrofolate dehydrogenase, NAD dependent (likely a methylene-tetrahydromethanopterin dehydrogenase in alkanotrophic archaea)
Mto: methoxyltransferase
Mtr: tetrahydromethanopterin *S*-methyl-transferase complex
Pta: phosphoacetyl transferase
Scr: short-chain alkyl–coenzyme M reductase complex. A subtype of Acr specialized in short-chain alkanes (e.g., butane, propane)

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REDUCTION/OXIDATION OF COFACTORS

Eha, Ehb, Ech, Ehd: energy-converting hydrogenase complexes
Fpo: $F_{420}H_2$-methanophenazine oxidoreductase complex
Fpo-l: ferredoxin-methanophenazine oxidoreductase-like complex
Fqo: $F_{420}H_2$-methanoquinone oxidoreductase complex
Frh: coenzyme F_{420}–reducing hydrogenase complex
GltD-l: glutamate synthase D-like protein
Hdr: heterodisulfide reductase complex (cytosolic, composed of subunits ABC)
HdrDE: heterodisulfide reductase complex (membrane bound)
Mbh: membrane-bound hydrogenase complex
MHC: multi-heme cytochrome
Mvh: F_{420}-nonreducing hydrogenase complex (cytosolic)
Nrf: cytochrome *c* nitrite reductase
Psr-l: polysulfide reductase-like protein
Rnf: ferredoxin:NAD^+ (or methanophenazine) oxidoreductase complex
Vho/Vht: F_{420}-nonreducing hydrogenase complex (membrane bound)

COFACTORS

CoA, CoB, CoM: coenzymes A, B, and M
F_{430}: Ni(I) F_{430} cofactor essential for Mcr
Fd: ferredoxin (Fd_{red}, reduced; Fd_{ox}, oxidized)
H_4MPT: tetrahydromethanopterin. An analogue of H_4MPT, tetrahydrosarcinapterin (H_4SPT), is present in *Methanosarcinales* and *Methanococcales*
MFR: methanofuran

A novel type of methanogenesis (alkanotrophic methanogenesis) has been recently proposed based on the first metagenome-assembled genomes (MAGs) of the "*Ca*. Methanoliparia," which encode Acr and Mcr as well as other core proteins for alkanotrophy and CO_2-reducing methanogenesis (**Figure 1*b*,*c***). Dismutation of multicarbon alkanes (15, 57), and possibly long-chain fatty acids (15), would be coupled with methanogenesis within one cell, contrasting with the current paradigm of archaeal/bacterial syntrophy in this process (39, 121). This proposal has been nicely confirmed experimentally from enrichments of "*Ca*. Methanoliparia," which showed that these archaea are able to use a large panel of alkanes, alkylcyclohexanes, and alkylbenzenes (with linear chains greater than 13 carbons) (124).

Finally, the substrate range of methanogens has also been greatly extended recently by the discovery of dismutation of various methoxylated coal compounds in *Methermicoccus shengliensis* (*Methanosarcinales*), which is carried out by the same core enzymes as methyl-dismutating methanogenesis but with specific methyltransferases (56, 69) (**Figure 1*b*,*c***).

These new results have significantly reshaped our view of the diversity of methane-related metabolisms and provided key insights into their functioning and societal impact. Due to their large phylogenetic distribution in the Archaea, several of these pathways may have an ancient origin and represent some of the earliest microbial metabolisms. These enzymes are directly involved in the oxidoreductions and transfers of the carbon substrates, regeneration of electron

transporters, and conservation of energy. Their great modularity likely facilitated the emergence of a wide range of metabolisms and allowed archaea to occupy unique environmental niches. This review presents the current state of knowledge on the diversity and evolution of these metabolisms and discusses their origin and evolutionary trajectories.

2. CONSERVATION AND VARIABILITY OF METHANE-RELATED PATHWAYS

Eight main pathways (**Figure 1*a***) are centered around Mcr and Acr. In all cases, the energy is conserved by the generation of a chemiosmotic gradient (based on Na^+ or H^+) that is exploited by a membrane ATP synthase to produce ATP from ADP. While the enzymatic steps directly involved in the oxidoreduction of the carbon substrates are mostly uniform in each pathway, there exists a stunning variability in the mechanisms of energy conservation, regeneration of electron transporters, and transmission of electrons to the final acceptor (**Figures 2** and **3**).

2.1. Methyl-CoM and Alkyl-CoM Reductases, the Central Enzymes

For methanogenesis, Mcr catalyzes the reduction of a methyl group linked to CoM (CoM-S-CH_3) using coenzyme B (HS-CoB), which leads to the formation of CH_4 and CoM-S-S-CoB [also called heterodisulfide or HDS (**Figures 2** and **3**)]. Even if this reaction is highly exergonic under standard conditions [$\Delta G^{\circ\prime} = -30$ kJ/mol (101)], it can be reverted in methanotrophic archaea. Similar to Mcr, which can only catalyze reactions involving methane, Acr seems specialized for a specific alkane chain length. Acr enzymes may thus be subdivided into ethyl-CoM reductase (Ecr) of "*Ca*. Argoarchaeum"/ "*Ca*. Ethanoperedens" on ethane (41), short-chain alkyl-CoM reductase (Scr) (58), and long-chain alkyl-CoM reductase (Lcr) in "*Ca*. Methanoliparia" (124). Mcr and Acr are composed of three subunits (McrABG/AcrABG) that are assembled in a dimer of heterotrimers (31, 41). The interface between the dimers forms the active site, which contains the Ni(I) F_{430} cofactor that is essential for catalysis (120). About 30 genes are almost ubiquitous in archaea possessing Mcr/Acr but are absent from other archaea (15). Some are involved in F_{430} cofactor biosynthesis [CfbA-E (76, 123)], Mcr/Acr activation [AtwA and possibly McrC (85)], or posttranslational modification of amino acids in the Mcr catalytic site [McmA (79), MamA (29, 67, 86), YcaO/TfuA (80)]. Others have an unknown function but may be very important for Mcr/Acr functioning, such as the 6 genes that form a unique genomic cluster conserved in most Mcr/Acr-bearing archaea (15).

2.2. CO_2-Reducing Hydrogenotrophic Methanogenesis

The CO_2-reducing hydrogenotrophic methanogenesis pathway is present mostly in Class I and Class II methanogens and can be divided into five central enzymatic steps (**Figures 1*b*** and **2*a***) (reviewed in 92). (*a*) CO_2 is reduced into formyl by a reduced ferredoxin (Fd_{red}) and bound to a methanofuran (MFR) by FwdABCDE(FGH) to produce MFR-CHO (12, 109). (*b*) The formyl group is then transferred from methanofuran to tetrahydromethanopterin (H_4MPT) by Ftr. (*c*) The formyl bound to H_4MPT is successively reduced into methenyl, methylene, and methyl by Mch, Mtd, and Mer (see the sidebar titled Terms and Definitions), leading to H_4MPT-CH_3. The reduced F_{420} cofactor ($F_{420}H_2$) is the electron donor for the reactions performed by Mtd and Mer, and its regeneration is performed by the FrhABG hydrogenase complex using H_2 as an electron donor (102). (*d*) The methyl bound to H_4MPT is transferred to CoM-SH by the Mtr complex (MtrABCDEFGH), which couples the exergonic ($\Delta G^{\circ\prime} = -30$ kJ/mol) transfer to the export of Na^+ ions across the membrane, producing an electrochemical gradient that is used by

the ATP synthase (38, 110). (*e*) Finally, CoM-S-CH_3 is reduced by HS-CoB in Mcr, producing CoM-S-S-CoB and CH_4.

The CoM-SH and HS-CoB must be regenerated from CoM-S-S-CoB, and a ferredoxin (Fd) must be reduced to provide electrons in the initial step of the H_4MPT-MBWL pathway (**Figures 1*b*** and **2*a***). In most Class I and Class II methanogens, and possibly in "*Ca*. Nezhaarchaeota," this reaction is performed by the flavin-based electron-bifurcating HdrABC-MvhADG complex using two H_2 as electron donors (48, 111). MvhA is a [NiFe] hydrogenase, HdrB is a CoM-S-S-CoB reductase, and HdrA contains the electron-bifurcating flavin and is responsible for Fd reduction. Using the electrochemical gradient, additional complexes (Eha, Ehb, Ech, and Mbh) can perform the exergonic reduction of Fd with H_2 to replenish Fd_{red} removed from methanogenesis by biosynthetic pathways or imperfect coupling in electron bifurcation (62). In *Methanosarcina*, the regeneration of CoM-SH and HS-CoB and the reduction of Fd rely instead on three membrane complexes [Vho (or Vht)/HdrDE/Ech] and methanophenazine, a membrane-soluble electron transporter (68, 103). Vho (hydrogenase) and HdrDE (CoM-S-S-CoB reductase) reduce CoM-S-S-CoB using H_2. Methanophenazine transfers the electrons between these two complexes. This exergonic reaction is associated with buildup of an electrochemical gradient that is used by the Ech complex to perform the endergonic reduction of Fd with H_2 (**Figure 2*a***).

Finally, several methanogens without cytochromes use formate instead of H_2 and CO_2 (**Supplemental Figure 2**). FdhAB oxidizes formate into CO_2 and generates $F_{420}H_2$ (8), both used by the H_4MPT-MBWL. The reduction of Fd and CoM-S-S-CoB by formate is done by an electron-bifurcating FdhAB-MvhD-HdrABC complex (27). Other electron donors for CO_2 reduction have been described, such as ethanol, isopropanol, 2-butanol, and Fe^{2+} (reviewed in 55).

Supplemental Material >

2.3. Acetoclastic Methanogenesis

Present in *Methanosarcinales* (Class II methanogens), acetoclastic methanogenesis comprises four central enzymatic steps (**Figures 1*b*** and **2*b***). (*a*) Acetate is first converted into acetyl-CoA, either by the low-affinity/high-activity AckA and Pta enzymes [e.g., *Methanosarcina* (59)] or by the high-affinity/low-activity Acs enzyme [e.g., *Methanosaetaceae* (10)]. Both systems require ATP. (*b*) The Cdh complex of the carbonyl branch of the Wood-Ljungdahl pathway (CBWL) converts acetyl-CoA into CO_2 and H_4MPT-CH_3 and reduces one Fd. The methyl group bound to H_4MPT is then (*c*) transferred to CoM-SH by Mtr (conserving energy) and (*d*) converted into CoM-S-S-CoB and CH_4 by Mcr.

Depending on the species, different sets of complexes can reduce CoM-S-S-CoB with the Fd_{red} generated by the CBWL pathway (Ech/Vho/HdrDE in *Methanosarcina mazei*, Rnf/HdrDE in *Methanosarcina acetivorans*, Fpo-I/HdrDE in *Methanosaetaceae*) (117) (**Figure 2*b***). This reaction is coupled to energy conservation by the buildup of the electrochemical gradient.

2.4. Methyl-Reducing Hydrogenotrophic Methanogenesis

The methyl-reducing hydrogenotrophic methanogenesis pathway is found across all Archaea [*Diaforarchaea*, *Methanotecta*, TACK, *Acherontia* (**Figure 1*c***; **Supplemental Table 1**)] and consists of two central enzymatic steps (**Figures 1*b*** and **2*c***). (*a*) The methyl group (CH_3-) of a substrate is first transferred to a corrinoid protein by a substrate-specific methyltransferase, MT1 (e.g., MtaBC) (55, 95), and then transferred to CoM-SH by a more generic methyltransferase, MT2 (e.g., MtaA) (**Supplemental Table 3**). (*b*) Mcr catalyzes the conversion of CoM-CH_3 and SH-CoB into CoM-S-S-CoB and CH_4.

Differently from CO_2-reducing and acetoclastic methanogenesis, the Mtr complex is not involved in energy conservation. Instead, a large diversity of mechanisms to conserve energy is predicted among methyl-reducing methanogens relying on the direct or indirect reduction of

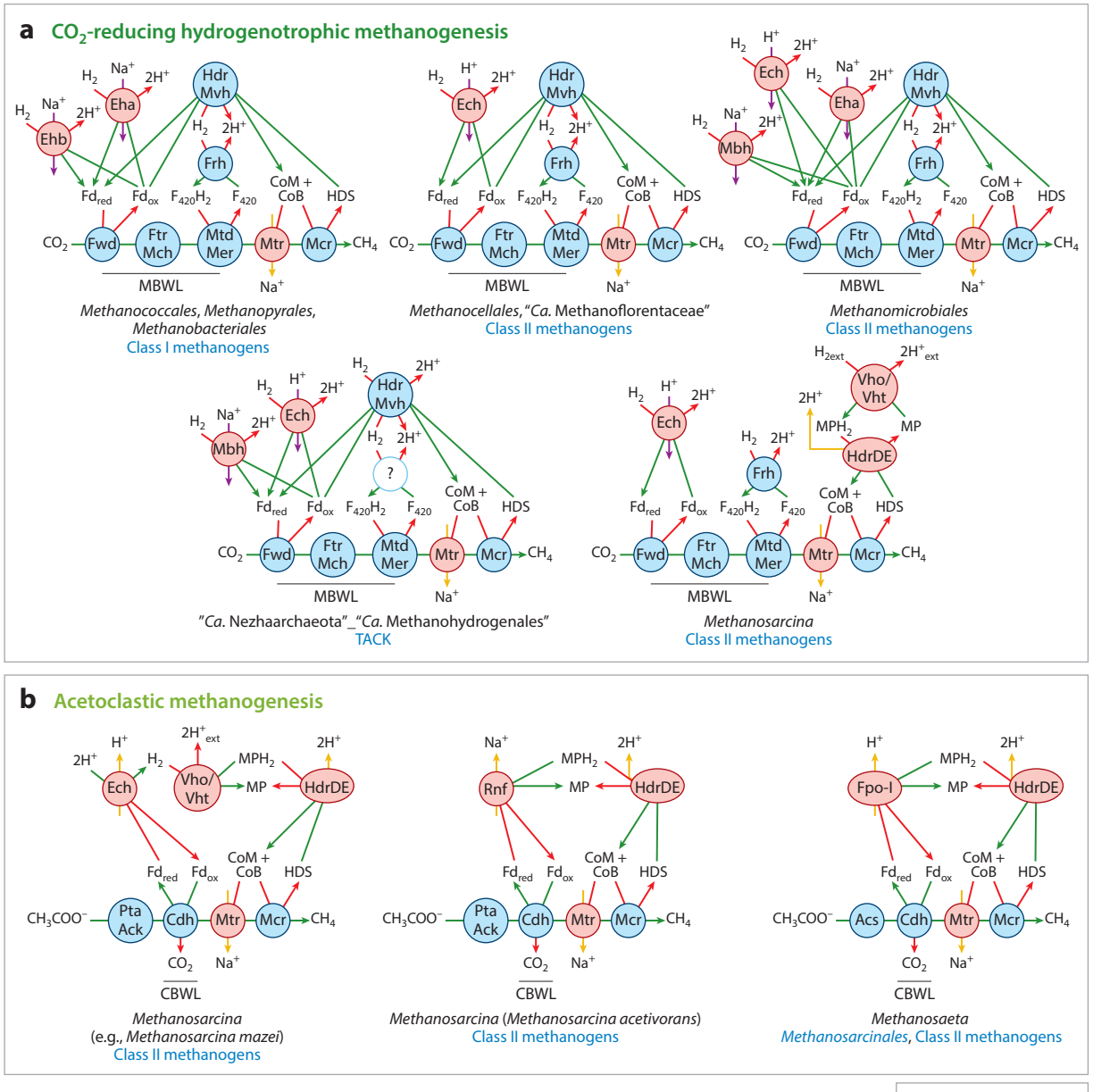
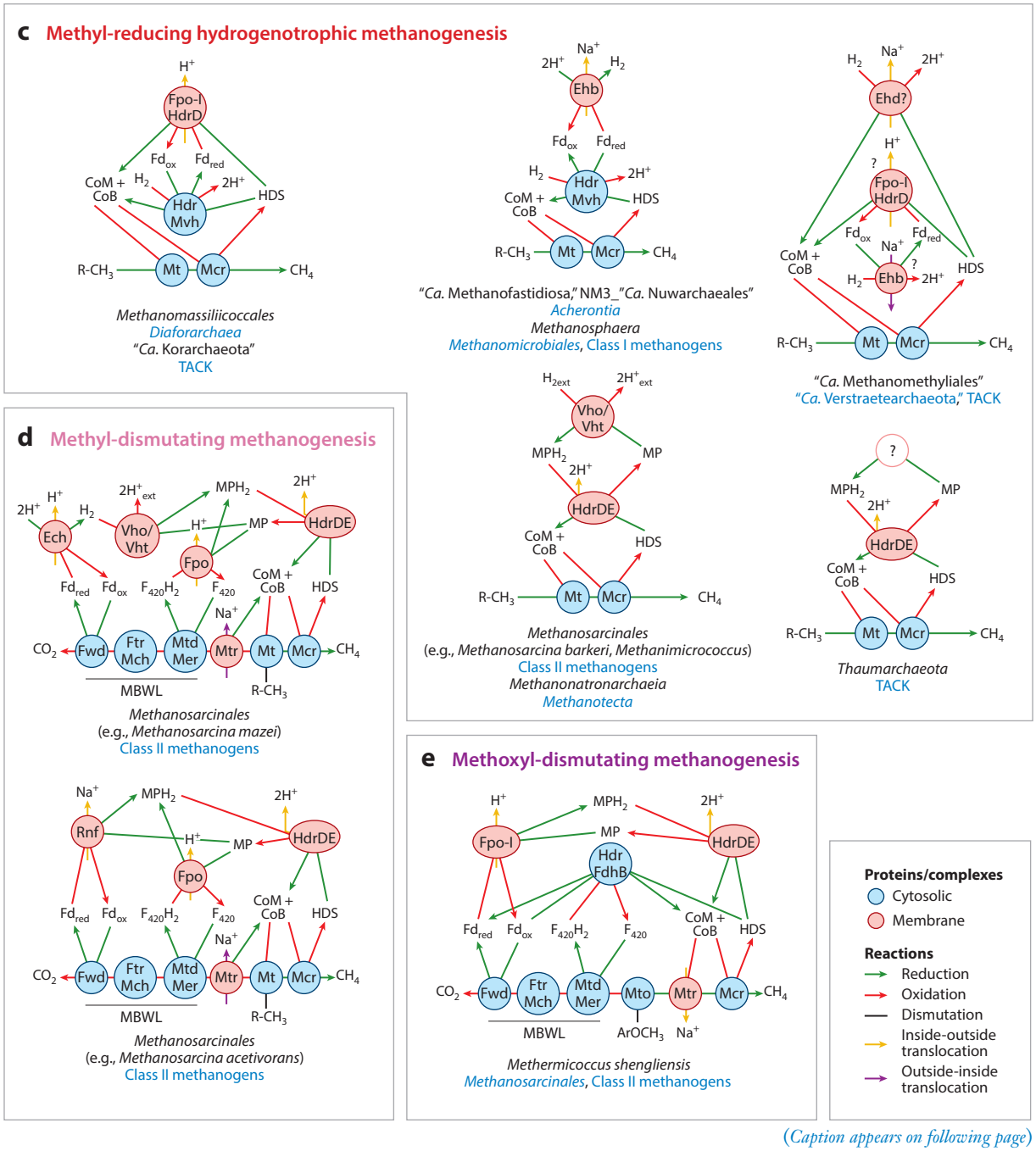


Figure 2

(*Continued*)

CoM-S-S-CoB by H_2 (**Figure 2*c***). In *Methanomassiliicoccales*, an HdrABC-MvhADG complex uses two H_2 to reduce CoM-S-S-CoB and Fd. The Fd_{red} is then used to reduce another CoM-S-S-CoB by the membrane Fpo-I complex likely associated with HdrD, coupling this reaction with energy conservation by translocating H^+ outside the cell (54). A similar mechanism may exist in "*Ca*. Methanodesulfokores" ("*Ca*. Korarchaeota"), but HdrB might be replaced by HdrD in the



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Diversity of mechanisms for regeneration of oxidoreduction states of electron transporters in (*a*) CO_2-reducing hydrogenotrophic methanogenesis, (*b*) acetoclastic methanogenesis, (*c*) methyl-reducing hydrogenotrophic methanogenesis, (*d*) methyl-dismutating methanogenesis, and (*e*) methoxyl-dismutating methanogenesis. Circles correspond to proteins and protein complexes and arrows to reactions. The color of each arrow corresponds to the type of biochemical reaction. The color of each circle indicates cellular localization. Enzymes of the MBWL and CBWL pathways are underlined. The steps that are not yet resolved are indicated by question marks. Names in blue are high-rank taxonomic groups, as in **Figure 1*b***. Ftr/Mch, Mtd/Mer, and Pta/Ack have been grouped together for brevity but are not known to form a complex. The other enzyme names grouped in the same circle are known or expected to form a complex. For expanded names of all abbreviated enzymes, see the sidebar titled Terms and Definitions. Abbreviations: CBWL, carbonyl branch of the Wood-Ljungdahl pathway; CoB, coenzyme B; Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin; HDS, heterodisulfide; MBWL, methyl branch of the Wood-Ljungdahl pathway; MP, methanophenazine; TACK, superphylum composed notably of *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and "*Ca.* Korarchaeota."

Hdr-Mvh complex (72). In *Methanosphaera*, "*Ca.* Methanofastidiosa," and "*Ca.* Nuwarchaeales," (NM3), an HdrABC-MvhADG complex also generates Fd_{red}. In this case, however, Fd_{red} is used by the membrane complex Ehb to reduce $2H^+$, which is coupled to translocation of Na^+ outside the cell (15, 81, 103). "*Ca.* Methanomethyliales," a proposed Ehd membrane complex (formed by HdrBC-Ech homologs), may directly reduce CoM-S-S-CoB with H_2 and couple this reaction to H^+ translocation in the extracellular space (15). In *Methanosarcina* and *Methanonatronarchaeia* (methanogens with cytochromes), CoM-S-S-CoB reduction by H_2 is performed by the Vho and HdrDE complexes (68, 96). In *Methanonatronarchaeia*, formate can be used as an electron donor to reduce methanophenazine, with the Fdn complex replacing Vho. Finally, the model of the pathway of *Thaumarchaeota* has not been fully resolved yet (47), but the taxonomic distribution of components and the literature suggest the use of a methanophenazine-based system involving HdrDE and a yet unknown reducer.

2.5. Methyl-Dismutating Methanogenesis

Methyl-dismutating methanogenesis is found only in *Methanosarcinales* (**Figure 1**). One methyl group is used as an electron donor to reduce three other methyl groups into methane (50) in four main steps (**Figure 2*d***). (*a*) The methyl group (CH_3-) of a substrate is transferred to CoM-SH by two methyltransferases, similar to what occurs in the methyl-reducing methanogenesis, as described above. (*b*) Then, one methyl group is oxidized into CO_2 using Mtr and the H_4MPT-MBWL pathway in the reverse direction than in CO_2-reducing methanogenesis. In this direction, methyl transfer by Mtr is energy-consuming (Na^+ translocated inside the cell) and the reactions of the H_4MPT-MBWL pathway reduce two F_{420} and one Fd. (*c*) Depending on the *Methanosarcina* species, the conservation of energy by generation of an electrochemical gradient can be achieved by two alternative sets of membrane complexes [HdrDE/Fpo/Rnf or HdrDE/Vho/Fpo (98)] reducing three CoM-S-S-CoB with two $F_{420}H_2$ and one Fd_{red}. (*d*) The three HS-CoB released from these reactions are used to reduce three CoM-CH_3 into CH_4.

2.6. Methoxyl-Dismutating Methanogenesis

Methoxydotrophic methanogenesis has been recently described in *M. shengliensis*, where methyl groups from various methoxylated coal compounds (R-O-CH_3) are dismutated into CO_2 and CH_4 (56, 69) (**Figure 2*e***). This pathway resembles methyl-dismutating methanogenesis, but the methyl group from the substrate is transferred to H_4MPT instead of CoM-SH, using the MtoABC methyltransferases/corrinoid protein (**Supplemental Table 2**). Thus, differently from methyl-dismutating methanogenesis, Mtr does not consume energy during methyl oxidation, and it conserves energy when the methyl is reduced into CH_4. The methyl transfer to H_4MPT thus brings thermodynamic efficiency to the overall reaction well above what is observed in other

Supplemental Material >

methanogens (and anaerobes) (56). It was suggested that the cell decreases this efficiency by alternating the direction of the H_4MPT-MBWL pathway, where part of the Fd_{red} and $F_{420}H_2$ would be used to reduce CO_2 instead of being used for energy conservation by the reduction of CoM-S-S-CoB (56), which is supported by the production of CH_4 from CO_2 during methoxydotrophic methanogenesis (69).

2.7. Methanotrophy

No isolates of ANME archaea are currently available; thus, most knowledge of how this metabolism works comes from field measurements, enrichment cultures, and genomic analyses (21, 53, 70, 106). In this pathway, methane is oxidized into CO_2 using Mcr, Mtr, and the H_4MPT-MBWL functioning in the reverse direction than in the CO_2-reducing pathway (**Figures 1** and **3*a***). Thus, (*a*) Mcr generates CoM-S-CH_3 and HS-CoB from CH_4 and CoM-S-S-CoB, (*b*) the methyl transfer by Mtr consumes energy (Na^+ entering the cell), and (*c*) the H_4MPT-MBWL pathway generates CO_2, $F_{420}H_2$, and Fd_{red}. Interestingly, in "*Ca*. Methanophagales" the Mer enzyme of the H_4MPT-MBWL pathway may have been replaced by MetFV (15, 99). Characterized MetFV are associated with the tetrahydrofolate (H_4F)-MBWL pathway and reduce H_4F-CH_2 into H_4F-CH_3 using NADH as an electron donor (23). MetFV may therefore oxidize H_4MPT-CH_3 into H_4MPT-CH_2 and may generate NADH in "*Ca*. Methanophagales."

All ANME have a gene cluster coding for a potential electron-confurcating HdrABC-MvhD-FhdB complex (15), which was proposed to generate $F_{420}H_2$ from the oxidation of Fd_{red}, CoM-SH, and CoB-SH (6, 73). The $F_{420}H_2$ derived from H_4MPT-MBWL and HdrABC-MvhD-FhdB can be used by either a membrane Fpo complex to reduce methanophenazine in ANME-2a and ANME-2c, or a membrane Fqo complex to reduce menaquinone in "*Ca*. Methanophagales" and "*Ca*. Methanoperedens." This reaction is coupled to energy conservation by generating a chemiosmotic gradient. In terrestrial ANME ("*Ca*. Methanoperedens," "*Ca*. Methanophagales" ANME-1-THS), reduced menaquinones transfer their electrons to different compounds. "*Ca*. Methanoperedens" can reduce NO_3^- with NarGH/HCOII, NO_2^- with Nrf, and Fe(III) and Mn(IV) using multi-heme cytochrome (MHC), which are nanowire-like proteins conducting electrons, and possibly a much wider range of electron acceptors (32, 43, 60, 65, 66). "*Ca*. Methanophagales" ANME-1-THS lacks MHC-VhoC but instead has a Psr-like protein, possibly able to reduce S^{2-} to H_2S by using electrons from reduced menaquinone (15). In the other ANME lineages living in marine environments, electrons from reduced menaquinone/methanophenazine are then transferred to sulfate-reducing bacteria using MHC. This process—called direct interspecific electron transfer (DIET)—has been supported by different approaches beyond the identification of MHC-coding genes (71, 116). As an alternative to DIET, it was proposed that soluble electron carriers such as H_2, formate (78), zero-valent sulfur (75), or methyl sulfide (77) could bring more energy to the syntrophic partner, but there is a lack of experimental evidence for this. It was also suggested that both DIET and low-potential soluble electron transporters could be used during syntrophy so that both methanotrophs and SRB obtain sufficient energy for their growth (21).

2.8. Alkanotrophy

The oxidation of short-chain alkanes for energy production based on Acr has been recently described (22, 40, 41, 58) and inferred in a growing number of lineages (**Figures 1** and **3*b***). Enrichment cultures have been obtained only for "*Ca*. Syntropharchaeales" and "*Ca*. Argoarchaeum"/"*Ca*. Ethanoperedens." In all archaea having this metabolism, the alkane is oxidized into alkyl-CoM (e.g., butane → butyl-CoM) by Acr (22, 40, 41, 58). Then, the alkyl is oxidized into acyl and transferred to CoA (e.g., butyl-CoM → butyryl-CoA) by unknown enzymes, for which candidates have been suggested (40, 57, 58). In ethane-oxidizing "*Ca*. Argoarchaeum" and "*Ca*.

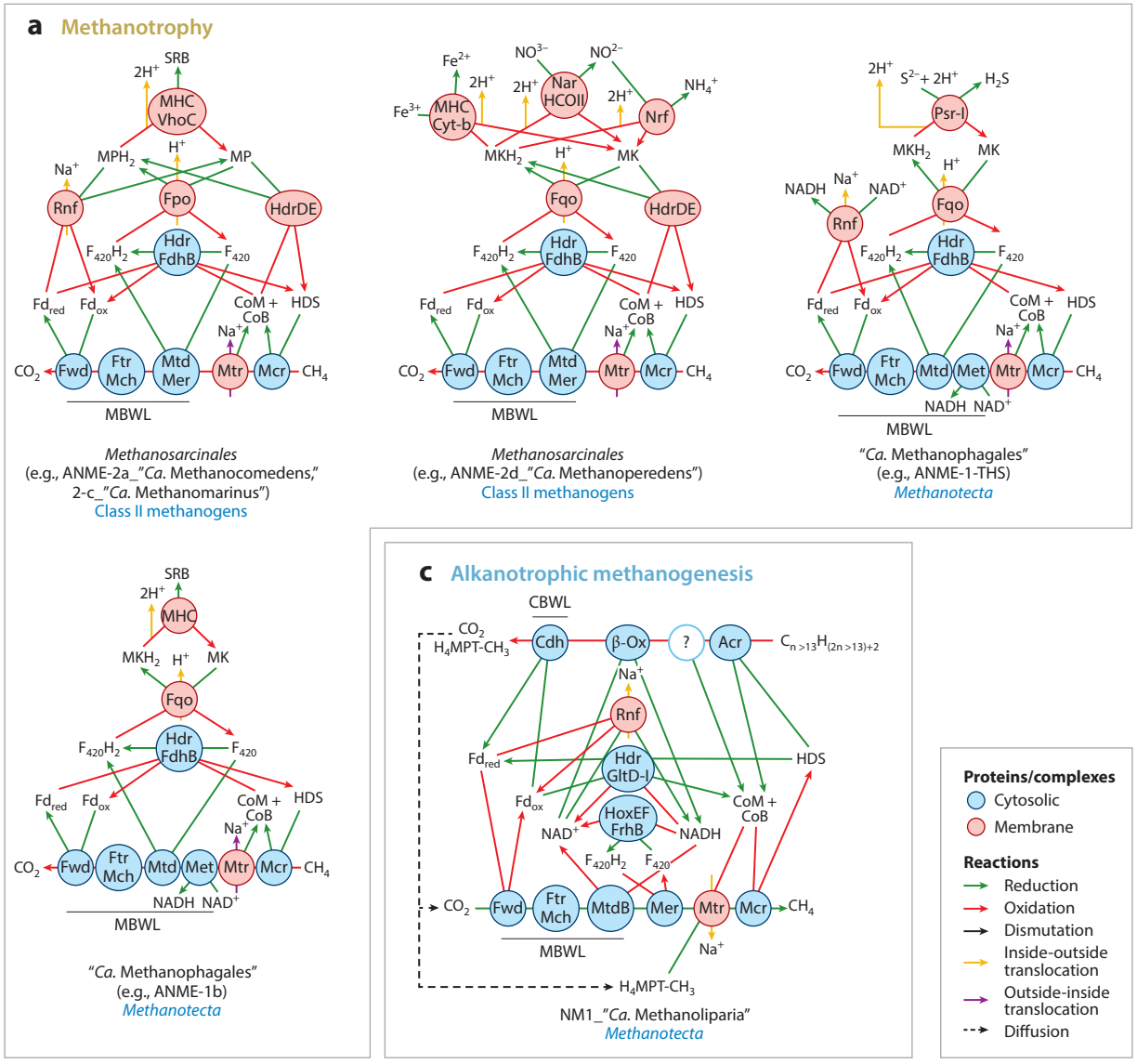


Figure 3

Diversity of mechanisms for regeneration of oxidoreduction states of electron transporters in (*a*) methanotrophy, (*b*) alkanotrophy, and (*c*) alkanotrophic methanogenesis. Circles correspond to proteins and protein complexes and arrows to reactions. The color of each arrow corresponds to the type of biochemical reaction. The color of each circle indicates cellular localization. Enzymes of the MBWL and CBWL pathways are underlined. The steps that are not yet resolved are indicated by question marks. Ftr/Mch, Mtd/Mer, and MtdB/Met have been grouped together for brevity but are not known to form a complex. The other enzyme names grouped in the same circle are known or expected to form a complex. For expanded names of all abbreviated enzymes, see the sidebar titled Terms and Definitions. Abbreviations: ANME, anaerobic methanotrophs; β-ox, β-oxidation; CBWL, carbonyl branch of the Wood-Ljungdahl pathway; CoB, coenzyme B; Cyt-b, cytochrome *b*; Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin; H_4MPT, tetrahydromethanopterin; HDS, heterodisulfide; MBWL, methyl branch of the Wood-Ljungdahl pathway; MK, menaquinone; MP, methanophenazine; Nar, nitrate reductase complex; SRB, sulfate-reducing bacteria.

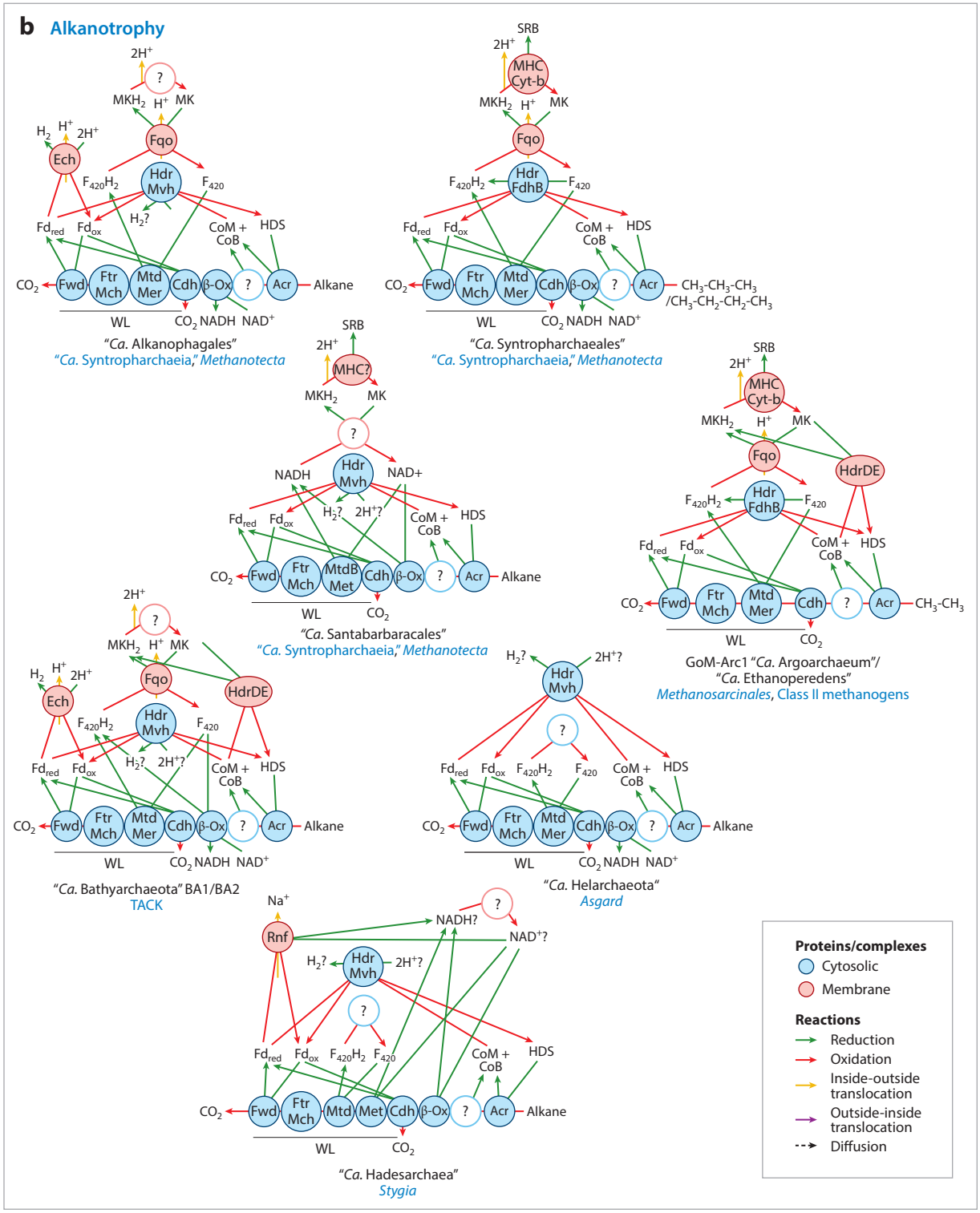


Figure 3

(*Continued*)

Ethanoperedens", these enzymes generate acetyl-CoA from ethyl-CoM (22, 40). In other lineages, the acyl-CoA (e.g., butyl-CoM) is further metabolized into several acetyl-CoA molecules by the β-oxidation pathway. In all Acr-bearing alkanotrophs, the acetyl-CoA is oxidized into CO_2 by the CBWL and H_4MPT-MBWL pathways. In the H_4MPT-MBWL pathway of "*Ca.* Santabarbaracales", Mer and Mtd are likely replaced by enzymes of the H_4F-MBWL pathway, MetFV and MtdB, respectively. The reoxidation of CoM-SH, HS-CoB, $F_{420}H_2$, NADH, and Fd_{red}, and its coupling with energy conservation, in "*Ca.* Syntropharchaeales" and "*Ca.* Argoarchaeum"/"*Ca.* Ethanoperedens" is similar with that in ANME. It may in fact involve HdrABC-MvhD-FhdB and Fqo complexes but also MHC-mediated DIET to sulfate-reducing bacteria (22, 40, 58). "*Ca.* Polytropus marinifundus" [(alternatively named "*Ca.* Allopolytropus marinifundi" (82a)] (*Archaeoglobales*) has been proposed to couple alkane oxidation with the reduction of various electron acceptors (oxidized metal, nitrogen, and sulfur compounds), directly or in association with bacteria through DIET (18)." All other lineages are missing one or several of these complexes, but they all encode a [NiFe]-hydrogenase (Mvh). Such a hydrogenase has been reported in "*Ca.* Helarchaeota" (91, 122), suggesting that electrons from alkane oxidation can be transferred via H_2 to a syntrophic partner that remains unknown because no enrichment culture is available.

2.9. Alkanotrophic Methanogenesis

It was recently revealed that alkanotrophy can be coupled to methanogenesis in "*Ca.* Methanoliparia" (15, 57, 124) (**Figure 3*c***). A range of long-chain alkanes (more than 13 carbons), as well as alkylcyclohexanes and alkylbenzenes, can be used by "*Ca.* Methanoliparia." This pathway is similar to other alkanotrophic pathways, up to the generation of CO_2, Fd_{red}, and H_4MPT-CH_3 by the CBWL pathway. At this point, H_4MPT-CH_3 is reduced to CH_4 through Mtr (conserving energy) and Mcr. To reoxidize the NADH and Fd_{red} generated by the β-oxidation and the CBWL pathway, part of the CO_2 produced by the CBWL pathway is reduced into CH_4 using the H_4MPT-MBWL pathway, Mtr, and Mcr. Thus, this alkanotrophic metabolism does not depend on direct or indirect (syntrophy) reduction of sulfate or other electron acceptors associated with methanotrophy and alkanotrophy. Accordingly, "*Ca.* Methanoliparia" cells do not form multispecies consortia (57, 124), and they do not encode genes involved in electron transfer to external acceptors (15, 57). This discovery changes the long-standing paradigm on the division of labor between bacteria and archaea for the methanogenic degradation of complex molecules. Many aspects of this novel metabolism remain to be elucidated, including the complexes involved in electron cycling [possibly Rnf, HdrABC-MvhD-GltD-like protein, HoxEF-FrhB, NfnAB (15, 57)].

3. EVOLUTION OF METHANOGENESIS AND METHANE-RELATED METABOLISMS

Methane-related pathways involve many enzymatic complexes that display high variability among archaea. Each complex is encoded by conserved genomic clusters, suggesting coevolution between subunits (3, 4, 15). The wide but patchy taxonomic distribution of methane-related enzymes suggests both a deep origin and a complex evolutionary history during archaeal diversification. Several analyses have been recently carried out to decipher the origin and evolution of methanogenesis and more broadly of methane-related pathways in Archaea (3, 4, 7, 11, 14–16, 33, 35, 47, 61, 114, 119).

3.1. When Did Methanogenesis Originate?

Since Mcr is the only known complex directly catalyzing the formation of methane, most studies addressing the evolution of methanogenesis rely on the phylogeny of one of its subunits (McrA) or a concatenation of the three (McrABG). An early study showed that Mcr sequences form two

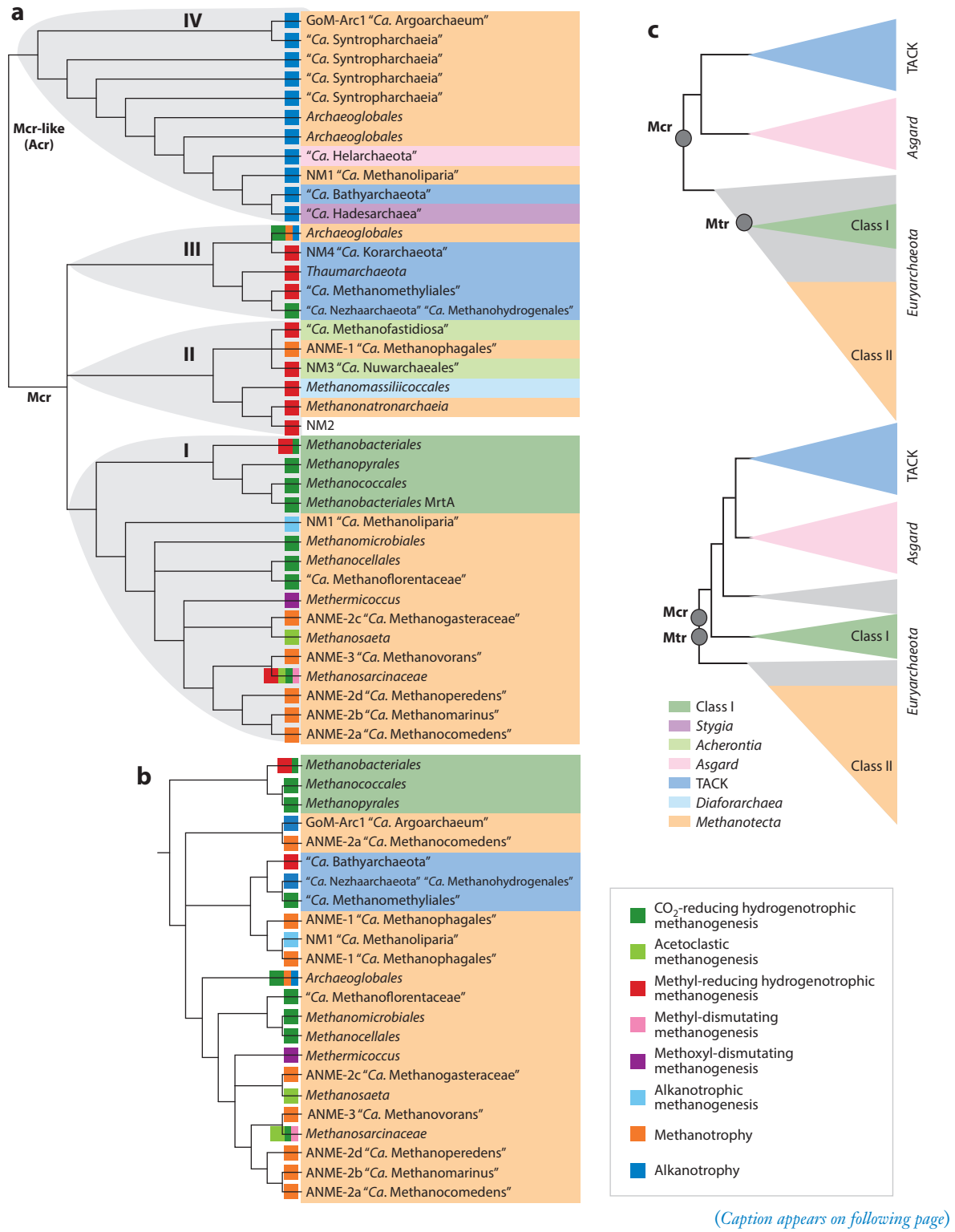
distinct clades corresponding to Class I and Class II methanogens, leading to the conclusion that Mcr originated in the *Euryarchaeota* and was then inherited vertically (7). More recently, culture-based and metagenomic-based analyses have uncovered Mcr homologs involved in methanogenesis, methanotrophy, and alkanotrophy (Acr) in a large number of novel lineages across the whole tree of Archaea, enriching the corresponding phylogeny (11, 15, 16, 18, 34, 47, 63, 81, 96, 108, 112, 114). A consensus of these analyses is shown in **Figure 4*a***. Currently, Mcr sequences cluster into four main groups, each corresponding to a type of methane metabolism: Group I is mainly linked to CO_2-reducing methanogenesis and groups II and III to methyl-reducing methanogenesis, and group IV (Acr) is associated with alkanotrophy (**Figure 4*a***). Some clades remain largely consistent with the archaeal phylogeny, notably Class I and II methanogens, and the TACK, implying that Mcr was present at least in the common ancestor of these clades (15, 114) (**Figure 4*c***). Some clear incongruences in groups II and IV suggest the occurrence of horizontal gene transfer (HGT) events. Moreover, the deepest relationships are not well resolved, probably due to the lack of phylogenetic signal at such taxonomic depth. For example, some studies have supported the monophyly of group II and III Mcr sequences (33) while others have not (15, 114). This makes it difficult to trace back with certainty the origin of the Mcr complex, and consequently of methane-related metabolisms in the Archaea. Moreover, interpretation of the Mcr tree strongly depends on where the root of the archaeal reference phylogeny lies, and especially the placement of the DPANN clade ("*Ca*. Diapherotrites," "*Ca*. Parvarchaeota," "*Ca*. Aenigmarchaeota," "*Ca*. Nanohaloarchaeota," and "*Ca*. Nanoarchaeota"). Some studies have proposed that DPANN are placed at the base of the archaeal tree, which would exclude an ancestral presence of methanogenesis in the last common ancestor of Archaea (LACA), as the DPANN lack the Mcr complex (**Figure 1*c***). Alternatively, the DPANN are episymbionts with drastically reduced metabolic capacities (19), and so the absence of Mcr may also be due to secondary loss. It has been proposed that the deep emergence of the DPANN might be a tree reconstruction artifact caused by their fast evolutionary rate and that they would have in fact emerged later (2, 84). Under this scenario, Mcr would be inferred as present in the LACA and consequently methanogenesis would be an ancestral trait in Archaea (**Figure 4*c***). This would fit with very early geological evidence of biological methane (3.5 Ga) (107) as well as molecular dating (114, 119).

Finally, the emergence of Acr is still unclear, as the clustering of the corresponding sequences in the phylogeny might be an artifact due to their high divergence following independent functional shifts from Mcr. Alternatively, Acr may have emerged from a duplication of *mcrABG* genes at the base of the "*Ca*. Syntropharchaeia" or *Methanotecta* and subsequently been transferred multiple times by HGT (15, 114).

3.2. What Is the Ancestral Type of Methanogenesis?

Despite current evidence for a likely early origin of the Mcr complex, the nature of the first type of methane metabolism is still unsettled. Based on the wide distribution of CO_2-reducing and methyl-reducing methanogenesis in the Archaea, the question mostly centers on which of these two types is ancestral to all present-day methanogens (11, 14, 15, 114). For this, the analysis of additional enzymes involved in either of these two metabolisms is key.

3.2.1. CO_2-reducing methanogenesis first? CO_2-reducing methanogenesis involves the enzymes of the H_4MPT-MBWL pathway, which are linked to Mcr by the Mtr complex (**Figure 1**). Bapteste et al. (7) proposed that in addition to Mcr, the H_4MPT-MBWL pathway and Mtr were also vertically inherited from the last common ancestor of Class I and Class II methanogens. Recent phylogenic analyses including novel lineages confirmed that the H_4MPT-MBWL pathway is ancestral in Archaea and evolved vertically (4, 118). Combined with the inference of a CBWL



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

(*a*) Schematic cladogram based on a consensus of currently available Mcr phylogenies. The roman numerals correspond to the major phylogenetic groups described in the text. (*b*) Schematic cladogram based on a consensus of currently available Mtr phylogenies. (*c*) Points of origin of Mcr and Mtr in Archaea according to two alternative roots, as presented by (*top*) Williams et al. (118) and (*bottom*) Raymann et al. (87). Abbreviations: Acr, alkyl–coenzyme M reductase complex; ANME, anaerobic methanotrophs; Mcr, methyl–coenzyme M reductase complex; Mtr, tetrahydromethanopterin *S*-methyl-transferase complex; TACK, superphylum composed notably of *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and "*Ca.* Korarchaeota."

pathway (Cdh) in the last universal common ancestor (3), this implies that the LACA had a complete Wood-Ljungdahl pathway, a strong argument in favor of CO_2-reducing methanogenesis being the first type of methanogenesis. However, the Wood-Ljungdahl pathway can work independently of methanogenesis [e.g., for CO_2 fixation, oxidation of acetyl-CoA produced by various catabolic pathways (52)], and several nonmethanogen lineages encode Wood-Ljungdahl pathway genes (4). Thus, the presence of both Mcr and Wood-Ljungdahl pathway enzymes in the LACA does not directly imply ability for CO_2-reducing methanogenesis. The origin of the Mtr complex is therefore of fundamental importance.

The most recent phylogeny of Mtr including sequences from TACK methanogens ("*Ca.* Methanohydrogenales"/"*Ca.* Nezhaarchaeota" and "*Ca.* Bathyarchaeota") shows that Mtr was acquired horizontally in separate events from Class I/II methanogens (114) (**Figure 4*b***). The authors thus proposed that Mtr emerged within *Euryarchaeota*. Nevertheless, another analysis with similar datasets reached opposite conclusions (present in the LACA) (5). This divergence is due to the uncertainties on the position of the root of the archaeal tree. Indeed, two different roots have been proposed, one between the TACK and *Euryarchaeota*, and the other within *Euryarchaeota* (87, 118) (**Figure 4*c***). Therefore, the answer to the question of whether Mtr—and consequently CO_2-reducing methanogenesis—was present in the ancestor of all methanogens strongly relies upon future analyses aimed at robustly resolving the root of the archaeal phylogeny.

3.2.2. Methyl-reducing methanogenesis first? Another candidate for the ancestral metabolism in Archaea is methyl-reducing methanogenesis. An early phylogenetic study of some of the methyltransferases and corrinoid proteins showed a clear monophyly of Class I and Class II, suggesting an ancestral presence in Archaea (16). However, the discovery of this type of metabolism in a growing number of TACK members (15, 16, 72, 81, 95, 96, 108), combined with their monophyly in Mcr trees (**Figure 4*a***), has reopened the issue. A recent phylogenetic analysis of the methyltransferases that transfer a methyl group from a corrinoid protein to CoM (MT2, i.e., MtaA, MtbA, and MtsA) concluded that these enzymes were likely present in the last common ancestor of the TACK and *Euryarchaeota* and, by extension, that methyl-reducing methanogenesis emerged early (114).

However, the evolutionary scenario of methyl-reducing metabolism might be much more complex. First, MT2 are part of very large protein families (56) and the associated phylogenies are difficult to infer and interpret reliably. Second, group II of the Mcr phylogeny includes methyl-reducing archaea from a mix of phylogenetically unrelated taxa, indicating potential acquisition of this metabolism by HGTs in these clades (**Figure 4*a***). Methyl-reducing methanogenesis could be more easily transferred than other types of methanogenesis, as it relies on only two components (Mcr and methyltransferases) that are adaptable to various energy conservation strategies (**Figure 2**). Moreover, most core methanogenesis genes are grouped in a unique gene cluster in some of these archaea (17) and could be transferred at once.

Supporting a late emergence, it has been recently proposed that methyl-reducing methanogenesis arose several times independently from other types of methanogenesis, in environments where it could confer a competitive advantage (105). This is the example of *Methanosphaera* and

Methanimicrococcus, two genera that are present almost exclusively in the animal gut and can only obtain energy through methyl-reducing methanogenesis, differently from their closest relatives not associated with a host (45, 74, 97, 105). Therefore, specialization in methyl-reducing methanogenesis likely occurred during adaptation to the gut environment (105). Indeed, the intestinal microbiome seems particularly propitious for this type of metabolism, as the methyl-reducing methanogens regularly constitute 10–100% of gut methanogens (104), whereas they are often less than 10% of the methanogens in open environments (28). In *Methanosphaera*, methyl-reducing methanogenesis likely emerged from CO_2-reducing methanogenesis (**Figure 5*a***) through loss of a few genes involved in the synthesis of a cofactor (molybdopterin) of the Fwd/Fmd complex (36). In contrast, in *Methanimicrococcus*, methyl-reducing methanogenesis likely emerged from methyl-dismutating methanogenesis (**Figure 5*a***) through the loss of most of the enzymes of the H_4MPT-MBWL pathway (105). Whether similar transitions toward rich environmental niches led to the very first emergence of methyl-reducing methanogenesis in earlier archaeal lineages remains to be studied further.

3.3. Origin and Evolution of Other Mcr-Associated Metabolisms

Another crucial issue is represented by the multiple transitions that led to the emergence of a wide diversity of metabolic pathways associated with the Mcr enzymatic complex (**Figures 1** and **5*a***). The phylogenetic distribution of these pathways involving Mcr or Acr (**Figure 1**) strongly suggests that they emerged relatively recently, possibly enabled by HGTs from bacteria or among archaea.

3.3.1. A burst of transitions within the *Methanosarcinales*. Most orders of methanogens are extremely homogeneous in the type of methane metabolism they use, being composed of either only CO_2-reducing or methyl-reducing members (**Figure 1**). One notable exception is *Methanosarcinales*, which display all main types of methane metabolisms as well as ethane oxidation (**Figures 1** and **5*b***). In association with Mtr and the Wood-Ljungdahl pathway, the cytochromes and membrane-soluble electron transporters constitute a versatile platform for electron transfer that is unique to this order and may have favored such diversification. Despite this large metabolic diversity, the majority of *Methanosarcinales* are either methyl-dismutating methanogens or anaerobic methanotrophs (**Figure 5*b***), likely corresponding to the most ancient metabolisms in this clade. The presence of methyl-dismutating methanogens in basal (*Methermicoccus*) and crown (*Methanosarcinaceae*) lineages suggests that this metabolism may have emerged first in *Methanosarcinales*.

Irrespective of the original type of methane metabolism in *Methanosarcinales*, transitions between methyl-dismutating methanogenesis and anaerobic methanotrophy may have occurred multiple times in this order (**Figure 5*a*,*b***), possibly facilitated by the environmental and metabolic relatedness of these two metabolisms. Indeed, both can thrive in marine sediments in the presence of sulfate: Most methanotrophic archaea are syntrophic partners of sulfate-reducing bacteria (53), and methyl-dismutating methanogens do not compete with sulfate-reducing bacteria for methyl-compound utilization, in contrast to other types of methanogens that are outcompeted for hydrogen and acetate (82). Moreover, in both methyl-dismutating methanogenesis and anaerobic methanotrophy, the Mtr complex and H_4MPT-MBWL pathway function in the oxidative direction, and most enzymatic complexes involved in methanotrophy are also involved in methyl-dismutating methanogenesis (**Figure 1**).

If both methyl-dismutating methanogenesis and anaerobic methanotrophy are ancient metabolisms in the *Methanosarcinales*, it is likely that acetoclastic methanogenesis, methoxyl-dismutating methanogenesis (56), and ethane oxidation (15, 22, 40) emerged more recently.

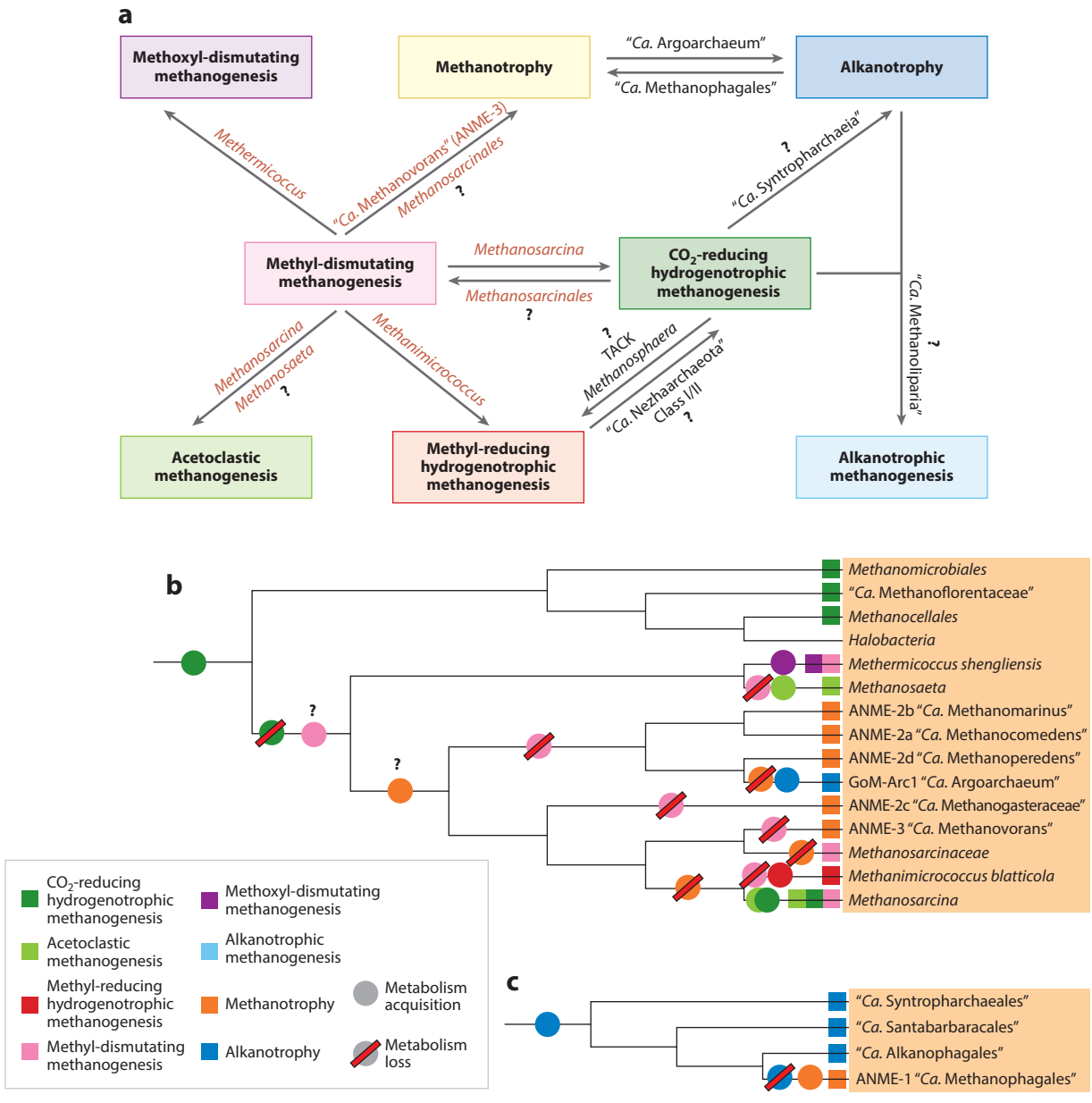


Figure 5

(*a*) Potential evolutionary transitions between different types of methane-related pathways. Names on arrows correspond to examples of clades in which the corresponding transition may have occurred. Names in salmon font correspond to transitions in the *Methanosarcinales*. (*b*,*c*) Proposed scenario for the diversification of pathways in the *Methanosarcinales* and the "*Ca*. Syntropharchaeia." Crossed-out circles represent loss of metabolism, and circles that are not crossed out indicate metabolism acquisition. Abbreviations: ANME, anaerobic methanotrophs; TACK, superphylum composed notably of *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and "*Ca*. Korarchaeota."

The late emergence of these metabolisms is consistent with the fact that they are present only in *Methanosarcinales* (**Figure 1**). Supporting this hypothesis, several HGTs potentially linked to these metabolic shifts have been identified. For example, the genes involved in methoxyl-dismutating methanogenesis in *M. shengliensis* were acquired from bacteria (56). Similarly, the phosphoacetyl-transferase (*pta*) and acetate kinase (*ackA*) genes involved in acetoclastic methanogenesis in *Methanosarcina* were acquired from *Clostridiales* around 240 ± 41 Ma (35, 89). The acquisition of acetoclastic methanogenesis by *Methanosarcina*, together with the increased availability of nickel (a limiting factor for methanogens) due to volcanism, is thought to have played a role in the end-Permian extinction that occurred in the same period (89).

The exceptional diversification of the possible electron acceptors for methanotrophy in "*Ca.* Methanoperedens" (ANME-2d) may also be the result of multiple HGTs (43, 61). Transfers from bacteria occurred for *narGH* genes needed for nitrate reduction (43), several molybdopterin oxidoreductase genes for reduction of other nonmetallic electron acceptors, and several menaquinone:cytochrome *c* oxidoreductases, as well as some of the MHC genes for the reduction of Fe(III), Mn(IV), or humic acids (61).

Finally, a potential HGT of Ecr to "*Ca.* Argoarchaeum"/"*Ca.* Ethanoperedens" (GoM-Arc1) was likely responsible for a shift from methanotrophy to ethane oxidation in this lineage (**Figure 5*a***). This transition from methanotrophy to ethane oxidation was inferred on the basis of the emergence of "*Ca.* Argoarchaeum"/"*Ca.* Ethanoperedens" within a clade consisting solely of methanotrophs [i.e., ANME-2d "*Ca.* Methanoperedens," ANME-2a "*Ca.* Methanocomedens," and ANME-2b "*Ca.* Methanomarinus" (15) (**Figure 5*b***)], and may have been eased by the very similar mode of energy conservation between these ethane and methane oxidizers (**Figure 3**).

3.3.2. Emergence of methane/multicarbon alkane metabolisms in the other archaeal lineages. The close phylogenetic relationship between anaerobic methanotrophs and short-chain alkane oxidizers is not unique to the *Methanosarcinales* but is also observed within the "*Ca.* Syntropharchaeia" (**Figure 5*c***), suggesting that transitions between these two metabolisms happened more than once. However, here the transition occurred from short-chain alkane oxidation to methanotrophy, i.e., in the opposite direction than in the *Methanosarcinales* (**Figure 5*a***). Two arguments support this hypothesis. First, the methanotrophic "*Ca.* Methanophagales" are part of a clade composed of only short-chain alkane oxidizers (114) (**Figure 5*c***). Second, "*Ca.* Methanophagales" acquired their *mcrABG* genes by HGT from methyl-reducing methanogens belonging to the *Acherontia* (**Figure 4*a***) and likely replaced the *acrABG* genes initially present (15).

Another interesting and complex case of transition between methane/multicarbon alkane metabolisms concerns the *Archaeoglobales*. This order is closely related to several lineages of methanogens/methanotrophs and until recently was thought to be exclusively composed of nonmethanogens. The H_4MPT-MBWL pathway and some Mcr/Acr-associated markers in these archaea were interpreted as remnants of past methane metabolism (7, 14, 15). Surprisingly, recent data have now shown that several members of this class have more than these remnants; several MAGs belonging to "*Ca.* Methanomixophus" encode, in fact, both Mcr and Mtr complexes, and it has been proposed that they are able to perform CO_2-reducing and/or methyl-reducing methanogenesis and/or methanotrophy (24, 63, 112). It was inferred that "*Ca.* Methanomixophus" acquired its Mcr by HGT, implying a loss in the last common ancestor of *Archaeoglobales* and a later reacquisition (63). The initial loss of methanogenesis at the base of this class may have been triggered by the acquisition of bacterial genes involved in sulfite reduction [*dsrAB* (51)]. Interestingly, the Mtr complex appears to have been vertically inherited in "*Ca.* Methanomixophus." The role of this Mtr in the absence of Mcr (before its reacquisition) is unknown, but several "*Ca.*

Bathyarchaeota" genomes do code for Mtr and not Mcr (44), suggesting that this complex may be used in nonmethanogenic pathways. Despite the close phylogenetic relationship with other lineages of methanogens and methanotrophs and the presence of many genes involved in these metabolisms, the evolutionary history of methane metabolisms in the *Archaeoglobales* remains unclear because metabolic pathways are only partially resolved. However, they represent an interesting model to define metabolic transitions associated with gains and losses of genes of methane metabolisms.

4. OUTLOOK AND CONCLUSIONS

The field of archaeal methanogenesis is perhaps among those that have witnessed the largest growth over the last decade. Huge knowledge gaps have been filled by the discovery of a wealth of new lineages with the genetic potential for methane-related metabolisms in poorly explored anaerobic niches. The large majority of these lineages remain uncultured, and our knowledge of their metabolic potential is therefore based uniquely on sequence analysis. Nevertheless, the validity of such inferences (15, 57) has already been shown by the characterization of the first members of these lineages, such as "*Ca*. Methanoliparia" (124) and "*Ca*. Argoarchaeum"/ "Ca. Ethanoperedens" (22, 40). As a matter of fact, metabolic reconstructions based on MAGs have helped guide these experimental studies. Future efforts should be placed on the isolation and functional analysis of additional members of these lineages, as well as understanding their roles in the environment.

Given the huge impact of methane-related metabolisms in the history of Earth up to the present day, understanding the complex evolutionary trajectories that have led to such huge diversity is of paramount importance. Recent phylogenetic studies strongly suggest that methanogenesis—and more broadly, methane-associated—pathways are very ancient, some likely dating back to the LACA. This implies that the current diversity of archaea may stem from loss or tinkering of such ancestral methane metabolisms. The multiple independent losses of methane metabolisms are thus key transitions in the evolution of Archaea, as they were the starting points of the colonization of a wide range of niches (14). However, no consensus has yet been reached on the evolutionary history of these metabolisms. Its resolution in fact strongly depends on our ability to analyze such ancient events and to interpret the resulting data. One priority will surely be that of correctly placing the root of the archaeal phylogeny, as this is key to inferring the point of origin and the nature of the most ancestral methanogenesis pathway. Nevertheless, analysis of available data has already started providing important pieces of information. For example, the hypothesis is gaining traction that at least anaerobic methanotrophy emerged late and multiple times independently in the Archaea, possibly triggered by the large availability of both methane and inorganic electron acceptors during the early Paleoproterozoic (20). These events could have contributed to the drop in methane in the atmosphere that caused the first major Earth glaciation event (115).

We are still far from understanding the huge complexity of methane-related pathways and their components. For example, the diversity of methane-related pathways does not concern only core enzymes but also the enzymatic complexes responsible for regeneration of cofactors/ferredoxins and conservation of energy. These protein complexes are of key importance in methane-related pathways, but their evolutionary histories and functions have not been thoroughly studied. This lack of information precludes a global view of the evolution of methanogenesis in Archaea and represents a priority of future studies. It is also a great methodological challenge given the patchy taxonomic distribution of these proteins, which suggests very complex trajectories. Moreover, genomic analyses have highlighted the existence of proteins that are associated with methane-related metabolisms but that have no predicted function or have never been experimentally characterized (15, 37). This pool of proteins likely includes new methanogenesis components that await further investigation.

The ancestry and current diversity of methane-related metabolisms argue for complex interactions with geological and ecological processes during the history of Earth that should be explored by future studies combining biochemistry, environmental microbiology, and evolutionary approaches. A better characterization of the unresolved methane/multicarbon alkane metabolisms will also be relevant to tackle major societal challenges associated with environmental sustainability, industrial applications, microbiomes, and health.

DISCLOSURE STATEMENT

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