Identification and characterization of a bacterial core methionine synthase

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Methionine synthases are essential enzymes for amino acid and methyl group metabolism in all domains of life. Here, we describe a putatively anciently derived type of methionine synthase yet unknown in bacteria, here referred to as core-MetE. The enzyme appears to represent a minimal MetE form and transfers methyl groups from methylcobalamin instead of methyl-tetrahydrofolate to homocysteine. Accordingly, it does not possess the tetrahydrofolate binding domain described for canonical bacterial MetE proteins. In Dehalococcoides mccartyi strain CBDB1, an obligate anaerobic, mesophilic, slowly growing organohalide-respiring bacterium, it is encoded by the locus cbdbA481. In line with the observation to not accept methyl groups from methyl-tetrahydrofolate, all known genomes of bacteria of the class Dehalococcoidia lack metF encoding for methylene-tetrahydrofolate reductase synthesizing methyl-tetrahydrofolate, but all contain a core-metE gene. We heterologously expressed core-MetECBDB in E. coli and purified the 38 kDa protein. Core-MetECBDB exhibited Michaelis-Menten kinetics with respect to methylcob(III)alamin (KM ≈ 240 µM) and L-homocysteine (KM ≈ 50 µM). Only methylcob(III)alamin was found to be active as methyl donor with a kcat ≈ 60 s⁻¹. Core-MetECBDB did not functionally complement metE-deficient E. coli strain DH5α (ΔmetE::kan) suggesting that core-MetECBDB and the canonical MetE enzyme from E. coli have different enzymatic specificities also in vivo. Core-MetE appears to be similar to a MetE-ancestor evolved before LUCA (last universal common ancestor) using methylated cobalamins as methyl donor whereas the canonical MetE consists of a tandem repeat and might have evolved by duplication of the core-MetE and diversification of the N-terminal part to a tetrahydrofolate-binding domain.

Methionine plays an essential role as proteinogenic amino acid in all domains of life, as an initiation amino acid in protein translation7 and as a precursor in the formation of cysteine, carnitine, taurine and lecithin8,9. Moreover, methionine can be converted to S-adenosyl-L-methionine (SAM)4, which represents an activated methyl group donor for many fundamental cellular processes5,6. The final step in methionine de novo synthesis, the methylation of homocysteine to methionine, is catalyzed by different types of methionine synthases including cobalamin-dependent (MetH) and cobalamin-independent methionine synthase (MetE). Some bacteria, e.g. Escherichia coli, possess genes for both enzymes7 and repress the expression of metE in the presence of vitamin B128. Homocysteine methylation in mammals is catalyzed by mammalian methionine synthases (mMS) similar to bacterial MetH9, betaine-L-homocysteine-S-methyltransferase (BHMT) or S-methyl-L-methionine-L-homocysteine-S-methyltransferase (also known as BHMT-2)10. Fungi and plants encode exclusively MetE11 or BHMT-212. All known methionine synthase types contain a zinc ion in the active site that is essential for homocysteine binding and methyl group transfer13.

MetH (EC 2.1.1.13) catalyzes the methyl transfer from 5-methyl-tetrahydrofolate-monoglutamate (5-methyl-THF-Glu) to homocysteine. MetH from E. coli is a large monomeric protein of 1,227 amino acids (136 kDa) and is composed of four functional domains14. In the catalytic cycle the methyl group of methylcob(III)alamin is transferred to homocysteine forming cob(I)alamin and methionine. Subsequently, cob(I)alamin is remethylated using 5-methyl-THF-Glu as the methyl group donor regenerating methylcob(III)alamin. For
reactivation of cob(II)alamin to methylcob(III)alamin, which is generated in a side-reaction approximately once in 2,000 turnovers\textsuperscript{15}, SAM is required\textsuperscript{16}.

Canonical MetE proteins (EC 2.1.1.14) are described as a family of zinc-containing metalloenzymes sharing no sequence similarity with MetH\textsuperscript{1,13}. They catalyze the methylation of homocysteine using 5-methyl-THF-Glu\textsubscript{n} (n ≥ 3) as methyl donor without the involvement of cobalamin. MetE in \textit{E. coli} is a protein of 753 amino acid residues (85 kDa) that is composed of two homologous parts connected by a linker region (Fig. 1b), suggesting that the domains have evolved by gene duplication of a sequence encoding a smaller protein of approximately 340 amino acid residues. The linker region contains the N-terminal domain (N-terminal domain) and the C-terminal domain (C-terminal domain).

Figure 1. Bioinformatic analysis of the core-MetECDBB from \textit{Dehalococcoides mccartyi} strain CBDB1. (a) Maximum-Likelihood phylogenetic tree of MetE representatives was generated with MEGA7\textsuperscript{67}. Multiple amino acid sequence alignments of full length with deletion of gaps (MUSCLE algorithm) were used to generate the tree. The analysis involved 39 amino acid sequences including the C-terminus of tandem-repeat methionine synthases (tr-MetE) from bacteria (brown colors) and yeast (green) as well as core-MetEs from archaea (blue colors), \textit{Chloroflexi} (red) and \textit{Clostridiales} (brown). The gene loci are in brackets. (b) The crystal structure of core-MetECDBB was calculated with the I-TASSER server\textsuperscript{65}. Overlay of the crystal structure of tr-MetE from \textit{Neurospora crassa} (PDB No. 4ZTX, grey) with the structural model obtained for core-MetECDBB from \textit{D. mccartyi} strain CBDB1 (green) was obtained with PyMOL\textsuperscript{66}. Core-MetECDBB matches the C-terminal part of tr-MetEECDH (C-score = –0.27) but lacks the N-terminal part and the linker region. (c) Amino acid sequence alignment of selected MetE proteins. The Zn\textsuperscript{2+} -binding site HXCXnC (red) is conserved in annotated tr-MetEs and also in core-MetE homologs. Core-MetECDBB: core-MetE from \textit{D. mccartyi} strain CBDB1; core-MetE\textsubscript{Deally}: core-MetE from \textit{Dehalogenimonas lykanthroporepellens}; core-MetE\textsubscript{MMKA}: core-MetE from \textit{Methanococcus maripaludis}; tr-MetE\textsubscript{DH10B}: tandem-repeat MetE from \textit{Escherichia coli} DH10B; tr-MetE\textsubscript{Sau}: tandem-repeat MetE from \textit{Staphylococcus aureus}.

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bic respiration ("organohalide respiration") with hydrogen as electron donor. Strain CBDB1 encodes one of the largest numbers of B$_{12}$-dependent proteins in known prokaryotes. The prominent representatives of this protein family are widespread in microorganisms with strongly conserved ancient traits (archaea, bacteria, and Chloroflexi sequences with high sequence similarity to core-MetECBD). The computational model indicates that in strain CBDB1, zinc is coordinated in a well separated from the C-termini of tr-MetE proteins and core-MetE Archaea (Fig. 1a, blue colors). Core-MetE proteins are widely distributed in microorganisms with strongly conserved ancient traits (archaea, Clostridiales, Dehalococcoidia and Chloroflexa classes). Within the archaea, only Haloquadratum spp. encode a tr-MetE homolog (Fig. 1a, blue colors). Bacterial and archaeal core-MetE form a paraplyetic group (excluding the tr-MetE sequences), probably evolved before LUCA (last universal common ancestor) and branched into two groups. Tr-METs appear to have evolved from archaeal core-MetE (e.g. core-MetE in Clostridium kluyveri and C. oryzae) (Fig. 1a).

### Results

Bioinformatic analysis of locus cbdbA481 in the genome of *D. mccartyi* strain CBDB1. *D. mccartyi* strains are able to synthesize methionine *de novo*, although *D. mccartyi* genomes do not contain gene homologs of *metE*, *metH*, *bhmt* or *bhtm*-2. In the KEGG (Kyoto Encyclopedia of Genes and Genomes) database several enzymes of the *Dehalococcoides* methionine metabolism are annotated. The loci cbdbA476 and cbdbA477 in the genome of strain CBDB1 are annotated as SAM synthetase (EC 2.5.1.6) and -adenosyl-L-homocysteine methyltransferase (core-MetE$_{ATMM}$) of the methanogenic archaea Methanobacterium thermoautotrophicum. In our study we provide biochemical and genetic evidence that the locus cbdbA481 encodes a novel type of bacterial methionine synthase that appears to be an anciently derived MetE-related methionine synthase obtaining its methyl group from an external corrinoid rather than from folate. Together with archaeal methylcobalamin:homocysteine methyltransferases and bacterial homologs the gene product of locus cbdbA481 forms a new group of basal methionine synthases, referred to as core-MetE in the following.

### Heterologous production and purification of core-MetE$_{CBDB}$. To study the function of core-MetE$_{CBDB}$ in detail, the recombinant protein was heterologously produced in *E. coli* and purified. First, production and purification attempts were conducted for a C-terminally Streptavidin-tagged core-MetE$_{CBDB}$ using affinity chromatography for purification. However, native polyacrylamide gel electrophoresis (PAGE) indicated misfolding.
Core-MetE CBDB catalyzes methionine formation with methylcob(III)alamin as methyl donor. The enzymatic activity of purified core-MetE CBDB was tested using methylcob(III)alamin as methyl donor and homocysteine as methyl acceptor. In the presence of core-MetE CBDB, the UV/Vis absorption spectrum of methylcob(III)alamin, exhibiting a characteristic maximum at 524 nm, successively changed over time due to the consumption of methylcob(III)alamin and formation of cob(I)alamin and cob(II)alamin, as indicated by the emergence of absorption features at 681 nm and 474 nm, respectively (Fig. 3a). In the absence of core-MetE CBDB or homocysteine, the UV/Vis spectrum of methylcob(III)alamin remained unchanged (Fig. 3b,c).

In order to exclude any methyltransferase activity due to impurities of the protein preparation, E. coli cell-free extract was also tested and did not show any activity (Fig. 3d). Finally, in addition to the photometric measurements, the formation of methionine ([M + H]⁺ = 150.0583 m/z) during the enzymatic reaction was verified via liquid chromatography-mass spectrometry (LC-MS) (Supplementary Figure 2b). In the following, core-MetE CBDB enzyme activity was monitored by measuring the increase of absorption at 681 nm (Fig. 3e) or the decrease of absorption at 524 nm (Supplementary Figure 2a). Kinetic parameters for core-MetE CBDB were determined using an enzyme concentration of 0.1 µM. At a constant D,L-homocysteine concentration of 2 mM and varying methylcob(III)alamin concentrations, methionine was formed with a Vₘₐₓ = 1664 ± 50 nkat mg⁻¹ and a Kₘ = 236 ± 3 µM for methylcob(III)alamin (Fig. 3e). When different D,L-homocysteine concentrations were used at a fixed methylcob(III)alamin concentration of 0.5 mM, a Vₘₐₓ = 1582 ± 11 nkat mg⁻¹ and a Kₘ = 98 ± 0 µM for D,L-homocysteine were estimated (Fig. 3f). Since methionine synthase is specific for L-homocysteine, the apparent Kₘ for L-homocysteine might be half of that for D,L-homocysteine. The maximum turnover number (kₜₐₜ) was calculated to be about 60 s⁻¹. The substrate specificity of core-MetE CBDB was investigated by replacing homocysteine with 2 mM cysteine, 2 mM glutathione or 2 mM dithiothreitol. Core-MetE CBDB did not show any activity towards these thiol analogs (data not shown).

Additionally, 5-methyl-THF-Glu₃ was tested as a methyl group donor for core-MetE CBDB instead of methylcobalamin (Fig. 4b). In the negative control and also in the presence of core-MetE CBDB, slow demethylation of 5-methyl-THF-Glu₃ occurred abiotically. The demethylation of 5-methyl-THF-Glu₃ in the negative control and in the presence of core-MetE CBDB was not linked to L-methionine formation (Fig. 4b(I)), while in the presence of tr-MetEEco, methionine was formed exhibiting a signal at [M + H]⁺ = 150.0583 m/z (Fig. 4b(II)).
Methionine synthase activity of core-MetECBDB was observed between pH 5.0 and 9.0, with an optimum between pH 6 and 6.5 (Table 1). The thermal stability of purified tr-MetEEco and core-MetECBDB were assessed by recording protein melting curves using nano differential scanning fluorimetry (nanoDSF). For tr-MetEEco, a melting temperature $T_m = 55.8 \pm 0.2 ^\circ C$ was determined. In contrast, the $T_m$ of core-MetECBDB was at $68.8 \pm 0.0 ^\circ C$ (Supplementary Figure 3).

Core-MetECBDB does not complement tr-metE-deficient E. coli in vivo. The examination of enzymatic activities of core-MetECBDB in vitro has limitations. However, we were not able to conduct in vivo mutagenesis studies with strain CBDB1 because Dehalococcoides species are not yet genetically accessible. In order to obtain insights into the physiological role of the cbdbA481 gene product in vivo, we tested whether a tr-metE-deficient E. coli strain could be complemented by core-MetECBDB. Therefore, we generated a tr-metE-deficient knockout strain of E. coli DH5α ($\Delta$metE::kan) that still contained the metH gene for the cobalamin-dependent MetH. This strain was not able to grow in medium without added cyanocobalamin (Supplementary Figure 4, red solid line), but grew when cyanocobalamin was supplemented (Supplementary Figure 4, red dotted line). Next, the growth behavior of the mutant strain carrying different complementation plasmids was investigated. Either the original tr-metE_Eco gene or the core-MetE CBDB nucleotide sequence, both under the control of an arabinose promoter, were provided. Growth experiments with these complementation strains showed that neither of the two strains grew without inducing gene expression by arabinose. After induction with arabinose, tr-metE_Eco was able to complement the $\Delta$metE strain as expected (Supplementary Figure 4, blue dotted line), while core-MetECBDB was not (Supplementary Figure 4, green dotted line). These results suggested that core-MetECBDB does not have the same physiological function as the canonical tr-MetE_Eco.

Discussion

Methionine and SAM have been suggested to belong to the most ancient molecules on earth and might have emerged within or even before the “RNA world”42–44. Although methionine appears to have a continued central metabolic role for more than three billion years, different routes for its biosynthesis have evolved. The biochemically conserved methionine pathway appears to be the product of an evolutionary patchwork involving diverse methionine synthases5. In our study, we identified a novel bacterial MetE-like methionine synthase in D. mccartyi strain CBDB1 that uses methylcobalamin as methyl donor instead of methylated tetrahydrofolate. Our results suggest that this enzyme is the basal form of canonical tandem-repeat MetE (tr-MetE) proteins with roughly half its size and without the domain duplication of canonical MetE proteins evolved to enable tetrahydrofolate binding.
Homologs of this short methionine synthase are encoded in the genomes of several deeply-rooting obligate anaerobic microorganisms from both prokaryotic domains, including all Dehalococcoidia and many Clostridia (e.g. Desulfitobacterium metallireducens, C. kluyveri, C. oryzae) as well as almost all archaea sequenced so far (Fig. 1a). We refer to this short monomeric MetE form as "core-MetE", because several lines of evidence support the idea that this enzyme is the ancestral form of MetE.

Figure 4. Core-MetECDB from D. mccartyi strain CBDB1 does not catalyze the formation of L-methionine with 5-methyl-THF-Glu3 as the methyl donor. (a) Reaction described for tr-MetE from E. coli (tr-MetEEco) catalyzing the methylation of L-homocysteine with 5-methyl-THF-Glu3 to form L-methionine and THF-Glu3. (b) Representative HPLC chromatograms of a chemical standard of 5-methyl-THF-Glu3 (blue), of reaction products after an enzyme activity assay containing 5-methyl-THF-Glu3, D,L-homocysteine and either core-MetECDB (red) or tr-MetEEco (green) or no enzyme (black). The peak at RT = 21 min represents dithiothreitol added to all reactions. In the presence of tr-MetEEco 5-methyl-THF-Glu3 (RT = 18 min) reacts to form THF-Glu3 (RT = 17.4 min). Slow demethylation of 5-methyl-THF-Glu3 to THF-Glu3 did occur in the negative control and also in the presence of core-MetECDB. To evaluate if this demethylation was linked to L-methionine formation, the products of the activity assays were analyzed by mass spectrometry. I) No L-methionine was formed in the presence of core-MetECDB. II) The product of tr-MetEEco was identified as L-methionine ([M + H]+ = 150.0583 m/z).

| pH  | MeCbl(III) consumption [µM min⁻¹] | relative activity [%] |
|-----|-----------------------------------|-----------------------|
| 5.0 | 20.53 ± 4.46                      | 82.0                  |
| 5.5 | 19.12 ± 3.23                      | 76.4                  |
| 6.0 | 25.05 ± 0.37                      | 100                   |
| 6.5 | 23.87 ± 1.41                      | 95.3                  |
| 7.0 | 18.96 ± 0.07                      | 75.6                  |
| 7.5 | 21.61 ± 0.41                      | 86.3                  |
| 8.0 | 18.08 ± 1.02                      | 72.3                  |
| 8.5 | 17.70 ± 0.88                      | 70.6                  |
| 9.0 | 14.23 ± 0.61                      | 56.9                  |

Table 1. Demethylation of methylcob(III)alamin (MeCbl(III)) catalyzed by core-MetECDB from D. mccartyi strain CBDB1 in the presence of D,L-homocysteine at different pH values.

on the N-terminal domain. Homologs of this short methionine synthase are encoded in the genomes of several deeply-rooting obligate anaerobic microorganisms from both prokaryotic domains, including all Dehalococcoidia and many Clostridia (e.g. Desulfitobacterium metallireducens, C. kluyveri, C. oryzae) as well as almost all archaea sequenced so far (Fig. 1a). We refer to this short monomeric MetE form as "core-MetE", because several lines of
evidence hint at its basal descendence including the lack of duplication, the exclusive presence in deeply rooting phylogenetic taxa, and the dependence on corrinoids, which are thought to be ancient cofactors as they participate in fundamental processes such as ribonucleotide reduction, the Wood-Ljungdahl-pathway and methane formation.

Compared with tr-MetEEco, core-MetECBDB is more stable towards pH changes and thermal denaturation. The turnover number of core-MetECBDB is very high in comparison to other methionine synthases such as tr-MetEEco with or E. coli MetH with . The activity of MetH is based on domain movements, which could contribute to the lower catalytic rate in comparison to a small monomeric core-E. The relatively slow conversion rate of tr-MetE proteins can be due to the poor methylation power of 5-methyl-THF-Glu as mentioned in Fig. 4a and the weak nucleophilicity of homocysteine at physiological pH . In tr-MetE and MetH, 5-methyl-THF must be activated for the nucleophilic attack by protonation at N . In both MetE and MetH, the nucleophilicity of homocysteine is enhanced by coordination with Zn that serves as Lewis acid. While in the “base-on” form the dimethylbenzimidazole (Dmbz) base of methylcob(III)alamin is coordinated to the cobalt center of the corrin ring, in the “base-off” mode Dmbz is dissociated from the cobalt. Stabilization of the transition state of methylcob(III)alamin in the “base-off” or “base-off/His-on” binding mode enable nucleophilic attack of homocysteine by weakening the Co-C bond and by reducing the thermodynamic barrier. Thus, only binding modes “base-off” or “base-off/His-on” enable methyl transfer from methylcob(III)alamin. However, the “base-off” mode of methylcob(II)alamin which is characterized by strong spectral changes namely, a significant blue shift in the UV/Vis spectrum and reduced intensity of the band, was not observed in our study (Fig. 2). It is difficult to precisely distinguish between the “base-on” and “base-off/His-on” form because, the UV/Vis spectra of them are very . The formation of methionine and cob(I)alamin (Fig. 3a) can only take place if methylcob(III) alamin and homocysteine are bound to core-MetECBDB in a stable and catalytically favorable configuration. Due to the minor spectral changes, we propose that methylcob(III)alamin is utilized by core-MetECBDB in the “base-off/His-on” binding mode. The “base-off/His-on” binding mode is found in many B-dependent proteins with a consensus motif where His represents the lower axial ligand replacing the Dmbz moiety. In our computational model of core-MetECBDB, His122 points towards the active site of the protein and probably belongs to a truncated B12-binding motif with the sequence HxxG, conserved among all Dehalococcoidia (Fig. 5).

The determined K -value of for methylcob(III)alamin of ~240 µM is likely much higher than the intracellular concentration of free methylcobalamin. Therefore, the physiological methyl donor might not be soluble methyl(III)cobalamin. We hypothesize that the physiological methyl donor is a corrinoid protein that directly interacts with core-MetECBDB. Needless to say, that inference of physiological characteristics from the determination of enzyme activity in vitro is limited. Examining the role of core-MetE in vivo could shed more light on the essentiality and functionality of the enzyme. However, genetic modification of strains is not possible yet.

The methyl group transferred by Dehalococcoides methionine synthase origins from exogenously supplied acetate, as has been shown by Zhuang et al. Acetate is activated in Dehalococcoides by acetyl-CoA synthetase (ACS) to acetyl-CoA. Acetyl-CoA is then cleaved to free coenzyme A, carbon monoxide (which leaves the cell) and a methyl group originating from the C2-atom of acetate. This reaction is catalyzed by acetyl-CoA decarboxylase/synthase (AcSB), an enzyme known mostly for its activity in the opposite direction for carbon fixation via the Wood-Ljungdahl pathway. In D. mccartyi strain CBDB1, AcSB represents the acetyl-CoA decarboxylase and AcscD a dimeric corrinoid iron-sulfur protein (CoFeSP) to which the methyl group from acetyl-CoA is transferred.
Zhuang et al. hypothesized that the methyl group is then transferred from AcsCD to tetrahydrofolate and from there to homocysteine, but Dehalococcoides neither encode the methyltransferases acsE, responsible for the methyl transfer from CoFeSP to tetrahydrofolate nor the classical metE/metH, responsible for methyl transfer from methyl-tetrahydrofolate to homocysteine (Fig. 6a,b) [32]. Our results can now explain these two gaps by hypothesizing that the methyl group from AcsCD/CoFeSP is directly transferred to homocysteine by core-MetECBDB instead of taking the diversion via tetrahydrofolate (Fig. 6c). This hypothesis would also explain the absence of carbon monoxide dehydrogenase in Dehalococcoides which would be needed if the Wood-Ljungdahl pathway was employed for CO₂ fixation. With the direct transfer of methyl groups from CoFeSP to homocysteine, the cells would be independent from methyl-tetrahydrofolate and indeed metF encoding methylene-tetrahydrofolate reductase (MTHFR) is missing in all Dehalococcoides genomes [27]. This might be an unusual pathway in extant microbiology but in our view could represent a very early evolutionary stage in which methyl metabolism could have been independent from folates. This view is supported by the fact that methionine, SAM, corrinoids and coenzyme A are conserved between archaea and bacteria but tetrahydrofolate/tetrahydromethanopterin are not [45,46].

Figure 6. Pathways of L-glycine and L-homocysteine methylation in Dehalococcoides species and hypothesized involvement of THF and corrinoid proteins. (a) Genes annotated to be involved in the incomplete Wood-Ljungdahl pathway encoded in D. mccartyi strain 195 and the respective homologous genes in other Dehalococcoides. (b) L-serine formation via glycine hydroxymethyltransferase (GlyA, grey) as proposed by Zhuang et al. is shown [32]. The methyl group is derived most probably from formate with the aid of formyl-tetrahydrofolate synthase (Fhs, yellow) and methylene-tetrahydrofolate dehydrogenase/cyclohydrolase (FolD, blue). (c) Methylation of L-homocysteine is conducted by core-MetECBDB encoded by the locus cbdbA481 (purple) in D. mccartyi strain CBDB1. The original source of the methyl group is acetate, which is activated to acetyl-CoA and then cleaved by acetyl-CoA decarbonylase (AcsB, green) into HSCO₂, carbon monoxide (CO) and a methyl group. The standard activity of AcsB is to transfer the methyl group to a corrinoid iron-sulfur protein complex (CoFeSP) AcsCD (red). We speculate that the methyl group is directly transferred from the CoFeSP to the core-MetECBDB (A481) for L-homocysteine methylation (dashed arrow) but this transfer could also be indirect via a yet unidentified participant.
Enzymes similar to the core-MetE identified in *Dehalococcoides* were also found in the majority of archaea (Fig. 1a, blue colors). *M. thermoautotrophicum* and other methanogens are described to encode methylcobalamin:homocysteine methyltransferase (core-MetE*)[^1][^2], a protein of 308 amino acids that is also homologous to the C-terminal part of tr-MetE proteins. *In vitro* experiments showed that core-MetE uses methylated corrinoids for the methylation of homocysteine, similar to what we now found for the core-MetECBDB. Schröder and Thauer concluded that soluble methylcobalamin is unlikely the physiological methyl group donor and hypothesized that a corrinoid protein with yet unknown function could play this role. The gene products of *MTH1124* or *MTH1156* were proposed as possible candidates. *MTH1156* encodes MtrH, a protein with sequence similarity to the 5-methyl-THF-Glu-binding domain of Meth (*26% identity*)[^3][^4][^5]. MtrH is part of the methyl-tetrahydromethanopterin-coenzyme M methyltransferase complex and catalyzes the methylation of cob(I)alamin to methylcobalamin (III)alamin using methyl-tetrahydromethanopterin as methyl group donor[^6]. In contrast to methyl-THF biosynthesis in *Dehalococcoides* strains, methanopterin biosynthesis, a functional equivalent to THF in archaea, is fully encoded in all methanogenic archaea[^7][^8][^9]. The methyl group of methionine in methanogenic archaea is derived from methyl-tetrahydromethanopterin[^10][^11], which might be the primary methyl donor of archaeal methylcobalamin:homocysteine methyltransferases.

In conclusion, our findings show that bacterial core-MetECBDB homologs together with archaeal core-MetE representatives form a basal group of methionine synthases using methylcobalamin in *vitro* as co-substrate. Due to the fact that organisms encoding core-MetE enzymes are slowly growing strict anaerobes with strongly conserved ancient traits, we speculate that the core-MetE homologs are similar to an ancient methionine synthase encoded already in the genome of a predecessor of LUCA and therefore basal to both archaea and bacteria. We speculate that such basal methionine synthases were active in the metabolism of ancient microorganisms using methylcobalamin-containing proteins as methyl donors. Core-MetE, as the first biochemically described bacterial representative of these core-MetE proteins resembling the methylcobalamin:homocysteine methyltransferase from *M. thermoautotrophicum*, Tr-MetE proteins appear to have evolved by duplications of core-MetE and subsequently acquired the capacity to bind folate at the N-terminal part. In our phylogenetic analysis tr-MetE clusters with archaean core-MetE genes (Fig. 1a).

### Materials and Methods

#### General

All chemicals were purchased from Sigma-Aldrich (Munich, Germany) or Carl Roth (Karlsruhe, Germany). Whenever methionine or homocysteine are mentioned in the text, the L-form is meant. Chemicals used for mass spectrometry were obtained in LC-MS grade from Jena (Jena, Germany). Restriction enzymes, DNA polymerase, DNA and protein standards were obtained from New England BioLabs (Frankfurt/Main, Germany). Oligonucleotides and sequencing services were provided by Seqlab (Göttingen, Germany). All oligonucleotide primers, plasmids and strains used in this study are listed in Supplementary Tables 1 and 2. Anaerobic experiments were performed in a COY glovebox (Grass Lake, USA).

#### Bioinformatics

The structural model of CdbdA481 (core-MetECBDB) was calculated using the I-TASSER server[^12][^13]. Broadly defined, the server aligns the template protein with proteins of similar folds or with super-secondary structures from the PDB library by LOMETS. The overlay of core-MetECBDB and tr-MetE from *N. crassa* was generated with PyMOL[^14][^15]. The amino acid sequences of tr-MetEs were trimmed approximately at the position 370. For the multiple sequence alignment and construction of the phylogenetic tree, only the C-termini of truncated tr-MetEs from bacteria, yeast and complete amino acid sequences of core-MetEs from archaea, *Chloroflexi* and *Clostridia* were used. MEGA[^16] was used to calculate multiple amino acid sequence alignments using the implemented MUSCLE algorithm with default settings[^17]. The evolutionary relationship between different methionine synthase amino acid sequences was inferred by using the Maximum Likelihood method based on the JTT matrix model[^18]. Evolutionary distances were computed using Poisson correction and are expressed as the number of amino acid substitutions per site[^19].

#### Construction of expression and complementation plasmids

Based on pBAD30, expression and complementation plasmids were generated as described in supplementary information. The resulting plasmids pBAD_MetE and pBAD_CdbdA481 were used for the complementation experiments as well as for the heterologous production and purification of tandem-repeat MetE (tr-MetE) from *E. coli* and core-MetECBDB from *D. maccartyi* strain CBDB1.

#### Production and purification of recombinant tr-MetE[^20] and core-MetECBDB

*E. coli* DH10B containing pBAD30_MetE or pBAD30_CdbdA481 was set up in Luria-Bertani (LB) medium containing 100 µg mL[^−1] ampicillin and grown overnight at 37 °C and 140 rpm. On the following day, 1% (v/v) of the overnight culture was used to inoculate fresh LB medium containing the appropriate antibiotic. The cultures were grown at 37 °C under agitation at 140 rpm until the OD[^600] reached 0.4–0.5. Then, the production of either tr-MetE[^21] or core-MetECBDB was induced by the addition of 0.05% (w/v) L-arabinose. Additionally, the medium was supplemented with 1 mM ZnSO[^4] and MetE and CdbdA481 were produced for 5 h at 37 °C and 140 rpm. Then, the cells were harvested by centrifugation and washed with 50 mM Tris-Cl, pH 7.5. Purification of tr-MetE[^22] and core-MetECBDB was performed under anaerobic conditions in an anaerobic chamber. Both enzymes were purified by anion exchange chromatography using a MonoQ 5/50 GL column connected to an AKTA purifier FPLC system (GE Healthcare Life Sciences) as described in detail in the supplementary information.

#### SDS-PAGE and native PAGE

The purity of core-MetECBDB and tr-MetE[^23] protein preparations was evaluated by 10% SDS-PAGE. In addition, the oligomeric state of both proteins was investigated via 10% discontinuous native PAGE[^24].
Protein identification from SDS-PAGE by LC-MS/MS. Qualitative identification of the purified core-MetECBD and tr-MetEEco proteins was conducted mass spectrometrically. Therefore, protein bands at the height of 38 kDa and 80 kDa were excised from 10% SDS-PAGE gels. Acetonitrile, 10 mM DTT and 100 mM iodoacetamide were used to destain, to reduce and to alkylate the proteins within the gel slices. Subsequently, the proteins were digested with 0.1 µg trypsin (Promega) at 37 °C for 18 h. The resulting peptides were extracted from the gel matrix with 50% (v/v) acetonitrile and 5% (v/v) formic acid and dried. The peptides were again dissolved in 10µL 0.1% formic acid and subsequently desalted using C18 ZipTip Pipette Tips (Merck Millipore) and dried in a vacuum centrifuge. Prior analysis, the peptides were resuspended in 20µL 0.1% formic acid. Samples were analyzed on an LC-MS/MS system composed of a nano-UPLC system (UltiMate 3000 RSLCnano System, Thermo Fisher Scientific) equipped with an Acclaim PepMap 100 75 µm × 25 cm C18 column and connected to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) via an electrospray ion source (TriVersa NanoMate, Advion). Sample volumes of 5µL were injected onto the column and separated applying a flow rate of 0.3µL min⁻¹ with the aid of a 60 min gradient from 3.2% to 44% acetonitrile in water containing 0.1% formic acid. The mass spectrometer was operated in positive-ionization mode. The spray voltage was set at 2.2 kV and an electron spray ionization source temperature at 220 °C. Full MS1 scans were obtained over a mass range of 300–2000 m/z and the resolution in the Orbitrap was set to 240,000. The most intense ions (threshold ion count above 5.0 × 10⁴) were selected for fragmentation with the quadrupole, setting the isolation window to 1.6 m/z. Ions were fragmented by ETciD (ETD reaction time 100 ms, CID collision energy 35%). The resulting fragment ion spectra were obtained achieved in the Orbitrap at a resolution of 60,000 and a maximum injection time of 120 ms.

Protein and peptide identification. The raw mass spectrometric data were converted to mgf-files using ProteoWizard MSConvert v3.072. The software SearchGUI (v3.3.5)73 and the OMSSA search algorithm were used for peptide identification. Mass spectrometric data were searched against the E. coli proteome database obtained from UniProt (Taxon identifier 316385). A precursor ion mass tolerance of 10 ppm was used at the MS1 level and up to two missed cleavages were allowed. The fragment ion mass tolerance was set to 0.2 Da for the Orbitrap for peptide identification. Mass spectrometric data were searched against the E. coli 50 mM KH₂PO₄/K₂HPO₄ (pH 7.2), 100 mM NaCl, and 10% glycerol. First, the UV/Vis spectrum of 10µM free methylcob(III)alamin was recorded. To assess the binding mode of methylcob(III)alamin to core-MetECBD, the protein solution was mixed with methylcob(III)alamin in a 1:1 stoichiometry (10 µM each). The UV/Vis spectra of free and bound methylcob(III)alamin were compared.

Enzyme activity assay with methylcob(III)alamin as methyl group donor. Enzyme activity assays were set up at dim light under strictly anoxic conditions. The standard enzyme assay mix contained 50 mM Tris/HCl (pH 6.5), 150 mM NaCl, 10% glycerol, 0.5 mM methylcob(III)alamin and 0.1 µM enzyme. After 5 min preincubation at room temperature, the reaction was started by the addition of 2 mM D,L-homocysteine. The reaction was photometrically monitored either at 524 nm indicating the consumption of methylcob(III)alamin (ε₅₂₄ = 6,200 M⁻¹ cm⁻¹) or at 681 nm indicating the formation of cob(I)alamin (ε₆₈₁ = 1,200 M⁻¹ cm⁻¹) (Fig. 2a and Supplementary Figure 2)75. Enzyme kinetics of core-MetECBD were performed at concentrations of either 0.5 mM methylcob(III)alamin or 2 mM D,L-homocysteine while the concentration of the second substrate was varied.

Synthesis of (6R,5S)-5-methyl-5,6,7,8-tetrahydropteroyltri-'γ-L-glutamic acid (5-methyl-THF-Glu₅). The synthesis of 5-methyl-THF-Glu₅ was accomplished from commercially available PteGlu₃ under anoxic conditions following the modified protocol of Yeo and Wagner76 and as described in detail in the supplementary information. 5-methyl-H₄PteGlu₃ was stored at −20 °C.

Enzyme activity assay with 5-methyl-THF-Glu₅ as methyl group donor. Enzyme assays were performed under strictly anoxic conditions. The standard assay was set up in 25 mM Tris/HCl (pH 7.2) or 50 mM KH₂PO₄/K₂HPO₄ (pH 7.2), 100 µM MgSO₄, 100 µM MnSO₄, 10 mM diithiothreitol (DTT), 2 mM D,L-homocysteine and 150 µM 5-methyl-THF-Glu₅. The reaction was started by the addition of 0.25 µM tr-MetEEco or core-MetECBD. After an incubation time of 60 min at 37 °C, the reactions were stopped by heat denaturation at 80 °C for 10 min, then centrifuged at 15,000 rpm for 5 min (Eppendorf Centrifuge 5424 R) and analyzed by HPLC. A negative control under same conditions without protein was run to evaluate abiotic transformation of 5-methyl-THF-Glu₅. 5-methyl-THF-Glu₅, other folate derivatives and PteGlu₅ were analyzed with a JASCO HPLC 2000 series system equipped with an Equisil BDS C18 column (250 × 4.6 mm, 5 µm; Dr. Maisch HPLC GmbH, Ammerbuch-Entringen/Germany) following the modified protocol of Patring77. The identities of PteGlu₅, 5-methyl-THF-Glu₅ and L-methionine were confirmed via liquid chromatography-mass spectrometry in direct injection mode.

Generation of E. coli knockout strain. The E. coli DH5α (ΔmetE::kan) knockout strain was generated using the Quick & Easy E. coli Gene Deletion Kit (GeneBridges GmbH, Heidelberg, Germany) according to the manufacturer’s protocol78. Hereby, metE gene in E. coli DH5α was replaced by a linear kanamycin cassette. The introduction of the kanamycin cassette allowed for screening for the knockout strain at 20 µg/mL −1 kanamycin agar plates.
In vivo complementation of *E. coli* DH5α (ΔmetE::kan) and cultivation procedure. The *metE*-deficient *E. coli* DH5α (ΔmetE::kan) strain was transformed with pBAD30 (negative control), pBAD30_MetE (positive control) or pBAD30_CbdbA481. The first subculture was grown in 5 mL LB medium with 100 µg mL⁻¹ ampicillin and 20 µg mL⁻¹ kanamycin at 37 °C and 140 rpm overnight. An inoculum of 1% (v/v) of the first subculture was then used to inoculate the second subculture of 5 mL M9 minimal medium supplemented with 1 mM MgSO₄ 0.1 mM CaCl₂ 10 µM FeCl₃/EDTA, 1.2 mM thiamine, 0.3 mM L-leucine, 0.4% (v/v) glycerol and 0.4 µM cyanocobalamin, that was grown at 37 °C and 140 rpm overnight. Then, several 10 mL-tubes of fresh M9 medium containing all supplements except cyanocobalamin were inoculated with 1% (v/v) of the second subculture. The following main cultures were set up:

(a) *E. coli* DH5α wild type with or without 0.4 µM cyanocobalamin,
(b) *E. coli* DH5α (ΔmetE::kan) with or without 0.4 µM cyanocobalamin,
(c) *E. coli* DH5α (ΔmetE::kan) + pBAD30_MetE with or without 0.4 µM cyanocobalamin and with or without 0.05% (w/v) L-arabinose,
(d) *E. coli* DH5α (ΔmetE::kan) + pBAD_CbdbA481 with or without 0.4 µM cyanocobalamin and with or without 0.05% (w/v) L-arabinose.

The main cultures were then incubated at 37 °C and 140 rpm and growth was monitored by measuring the OD₆₀₀. *E. coli* DH5α still encodes the arabinose operon. However, arabinose at a concentration of 0.05% (w/v) sufficed to induce the production of core-MetECBDB and tr-MetE_EOF. In our experiments, reciprocal metabolism leads to preferential use of glycerol instead of arabinose.

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Author contributions
L.A. and D.D. conceived the study. D.D., R.H., G.L. and S.S. designed the experiments in coordination with L.A. R.H., D.D. and S.S. conducted the lab experiments. R.H. and D.D. analyzed the data. D.D. and L.A. wrote the manuscript, G.L. revised it.

Competing interests
The authors declare no competing interests.

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