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Characterization of an *O*-Demethylase of *Desulfitobacterium hafniense* DCB-2

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Besides acetogenic bacteria, only *Desulfitobacterium* has been described to utilize and cleave phenyl methyl ethers under anoxic conditions; however, no ether-cleaving *O*-demethylases from the latter organisms have been identified and investigated so far. In this study, genes of an operon encoding *O*-demethylase components of *Desulfitobacterium hafniense* strain DCB-2 were cloned and heterologously expressed in *Escherichia coli*. Methyltransferases I and II were characterized. Methyltransferase I mediated the ether cleavage and the transfer of the methyl group to the superreduced corrinoid of a corrinoid protein. *Desulfitobacterium* methyltransferase I had 66% identity (80% similarity) to that of the vanillate-demethylating methyltransferase I (OdmB) of *Acetobacterium dehalogenans*. The substrate spectrum was also similar to that of the latter enzyme; however, *Desulfitobacterium* methyltransferase I showed a higher level of activity for guaiacol and used methyl chloride as a substrate. Methyltransferase II catalyzed the transfer of the methyl group from the methylated corrinoid protein to tetrahydrofolate. It also showed a high identity (~70%) to methyltransferases II of *A. dehalogenans*. The corrinoid protein was produced in *E. coli* as cofactor-free apoprotein that could be reconstituted with hydroxocobalamin or methylcobalamin to function in the methyltransferase I and II assays. Six COG3894 proteins, which were assumed to function as activating enzymes mediating the reduction of the corrinoid protein after an inadvertent oxidation of the corrinoid cofactor, were studied with respect to their abilities to reduce the recombinant reconstituted corrinoid protein. Of these six proteins, only one was found to catalyze the reduction of the corrinoid protein.

Acetogenic bacteria were the first anaerobes described to utilize phenyl methyl ethers as energy substrates (2). These organisms mediate the cleavage of the substrate ether bond and utilize the methyl group, which is oxidized to CO_2 in the oxidative part of catabolism. The reducing equivalents derived from methyl group oxidation are transferred to CO_2 upon the formation of an enzyme-bound carbon monoxide, which is then combined with further methyl groups to finally yield acetate (6). The key enzymes in the methylotrophic phenyl methyl ether metabolism of acetogens such as *Acetobacterium dehalogenans* (23) and *Moorella thermoacetica* (13) are the *O*-demethylases. These inducible enzyme systems mediate the ether cleavage and transfer of the methyl group to tetrahydrofolate (FH_4). Until now, three of these acetogenic *O*-demethylase systems were purified and characterized (7, 14, 25). In general, they consist of four protein components: two methyltransferases (MTs) (MT I and MT II), a corrinoid protein (CP), and an activating enzyme (AE). Both MTs and CP are involved in the catalytic cycle (Fig. 1). MT I binds the methoxylated substrate and catalyzes the cleavage of the substrate's ether bond and the transfer of the methyl group to the superreduced corrinoid cofactor of CP ($[Co^I]$). The methyl group is further transferred from CP to FH_4 by MT II. In this reaction, methyl-FH_4, as an intermediate of the acetyl coenzyme A (acetyl-CoA) pathway, is formed. Due to the low redox potential of the $[Co^{II}]/[Co^I]$ couple, an inadvertent oxidation of the superreduced corrinoid cofactor may occur, and inactive $[Co^{II}]$-CP is formed. The reactivation, i.e., the reduction to the active $[Co^I]$-CP, is mediated by AE in an ATP-dependent reaction (Fig. 1). In *A. dehalogenans*, the genes encoding MT I, MT II, and CP are usually organized into an operon (31). Only one AE gene has been detected so far; this gene is not part of an *O*-demethylase operon. The corresponding protein is presumably involved in the reactivation process of all CPs involved in *O*-demethylation (31). The AE of *A. dehalogenans* is a member of the COG3894 protein family. It was shown previously that genes encoding COG3894 proteins are found in bacteria and archaea that catalyze corrinoid-dependent methyl transfer reactions (8, 31). Hence, it was concluded "that the COG3894 genes encode 'activases', which are Fe/S proteins mediating the activation and reduction of protein-bound corrinoids" (31).

Similar corrinoid-dependent methyltransferase systems have been described to be present in methanogenic archaea (29, 30). These enzyme systems enable the utilization of methyl substrates such as methanol, methylated amines, or dimethylsulfide (16, 34). The operon structure is similar to that of known *O*-demethylase operons (31); also, COG3894 protein-encoding genes were detected (8). In these methanogenic methyltransferases, the methyl group is transferred to coenzyme M.

Desulfitobacteria are most commonly described as reductively dehalogenating bacteria (12, 35). The *O*-demethylation was proposed previously to be an intermediary reaction in the dehalogenation of methoxylated chlorinated aromatics (5, 24). In 2004, it was shown that two strains of *Desulfitobacterium hafniense* (strains DBC-2 and PCE-S) were able to utilize a variety of phenyl methyl ethers as growth substrates when an appropriate electron acceptor (e.g., fumarate) was provided (26). CO_2, which is used as an electron acceptor in acetogens, cannot replace fumarate in *D. hafniense*, although all enzymes (including the carbon monoxide dehydrogenase) of the acetyl-CoA pathway involved in acetyl-CoA formation from CO_2 were active (18).

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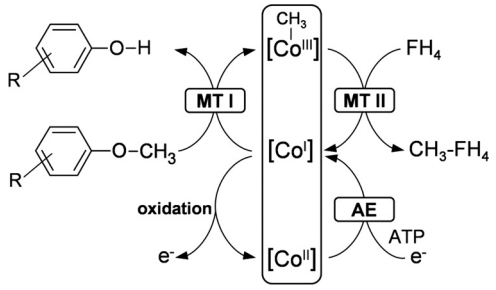

FIG 1 Scheme of the *O*-demethylase reaction in *Acetobacterium dehalogenans* (31). MT I, methyltransferase I; [Co^{I-III}], corrinoid protein with cobalt in the respective oxidation state; MT II, methyltransferase II; AE, activating enzyme; FH_4, tetrahydrofolate.

In this study, we screened the genome of *D. hafniense* DCB-2 (17) for corrinoid-dependent methyltransferases for the further characterization of an *O*-demethylase enzyme system. The heterologous expression of putative *O*-demethylase genes of *D. hafniense* DCB-2 in *Escherichia coli* is described. The corresponding recombinant proteins were characterized with respect to their possible function as MT I, MT II, CP, or AE.

MATERIALS AND METHODS

All chemicals, biochemicals, and gases needed for the growth of microorganisms and protein purification were of the highest available purity and were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), Fermentas GmbH (St. Leon-Rot, Germany), AppliChem GmbH (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany), IBA GmbH (Göttingen, Germany), and Linde AG (Pullach, Germany). Enzymes for molecular biology, if not stated otherwise, were purchased from Fermentas GmbH (St. Leon-Rot, Germany).

Cultivation of *Desulfitobacterium hafniense* DCB-2 and isolation of genomic DNA. *D. hafniense* DCB-2 was grown on pyruvate-fumarate (40 mM each) as described previously (26). The cells were harvested by centrifugation for 10 min at 8,000 × *g* and at 10°C. The genomic DNA was isolated according to methods described previously by Bollet et al. (3). Note that after cell disruption with SDS at 65°C, the genomic DNA was in the supernatant.

Construction of expression cassettes and cloning of the genes of the putative *O*-demethylase components. Expression cassettes for the genes Dhaf_1265, Dhaf_2573, Dhaf_2795, Dhaf_3310, Dhaf_3879, Dhaf_4322, Dhaf_4610, Dhaf_4611, and Dhaf_4612 (GenBank accession no. CP001336.1) as Strep tag fusions (at the 3′ end) and with the restriction sites for cloning into pET11a (Agilent Technologies Sales & Services GmbH & Co. KG, Waldbronn, Germany) were constructed from PCR products. For amplification, two PCR steps were performed. In step 1, the gene of interest, the 5′ restriction site, and a part of the Strep tag sequence were amplified. Genomic DNA of *D. hafniense* DCB-2 was used as the template. In step 2, the Strep tag sequence was completed, and the 3′ restriction site was introduced. The PCR product of step 1 was used as the template for the second PCR. The PCR mixtures contained 50 ng of DNA, 25 pmol each primer, 60 mM Tris-HCl (pH 8.5), 25 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, 200 μM deoxynucleoside triphosphates (dNTPs), and 2.5 U of *Taq* DNA polymerase (Segenetic, Borken, Germany) in a final volume of 25 μl. After an initial denaturation step of 2 min at 95°C, 35 cycles of 1 min at 95°C, 45 s at 55°C, and 1 min (Dhaf_4610, Dhaf_4611, and Dhaf_4612) or 3 min (all other genes) at 72°C were performed. After the last cycle, a final elongation step of 10 min at 72°C was performed. The primer sequences are given in Table 1.

PCR products and pET11a were digested with NdeI and BamHI according to the manufacturer's protocol. For Dhaf_4322, a compatible BglII restriction site instead of BamHI was used. The ligation was performed at 22°C for 1 h in a solution containing 40 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 mM ATP, and 5 U of T4 ligase in a final volume of 20 μl. The reaction was stopped by heat inactivation at 65°C for 10 min.

The plasmids were transformed into *Escherichia coli* XL1-Blue (Agilent Technologies Sales & Services GmbH & Co. KG, Waldbronn, Germany) using heat shock. For the detection of clones that contain the insert, plasmids were isolated by using the GeneJET plasmid miniprep kit (Fermentas GmbH, St. Leon-Rot, Germany). Plasmids that showed the expected restriction pattern were used for sequencing (GATC Biotech AG, Konstanz, Germany).

Heterologous expression of the genes of the putative *O*-demethylase components in *Escherichia coli*. The production of recombinant proteins was performed with LB medium (5 g yeast extract, 10 g tryptone, 10 g NaCl per liter) containing the required antibiotics. Gene expression was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG), as indicated in Table 2. After induction, the cells were harvested by centrifugation for 10 min at 10,000 × *g* and stored at −21°C.

Purification of heterologously expressed Dhaf_4610, Dhaf_4611, and Dhaf_4612 gene products. The Dhaf_4610 and Dhaf_4612 gene products were purified by affinity chromatography on *Strep*-Tactin in a one-step protocol. Soluble cell extracts were obtained by the disruption of *E. coli* cells in a French pressure cell, followed by 15 min of centrifugation at 16,000 × *g* at 10°C. About 1 ml of crude extract (5 to 10 mg protein per ml) was mixed with an equal volume of 100 mM Tris HCl (pH 8.0) containing 150 mM NaCl. The cell extract was applied onto a 1-ml *Strep*-Tactin Superflow cartridge (IBA GmbH, Göttingen, Germany) preequilibrated with 10 column volumes of Tris buffer with 150 mM NaCl. The purification of tagged proteins was performed according to the manufacturer's protocol.

E. coli expression strain BL21(DE3) is not able to synthesize vitamin B_{12}. Therefore, upon induction, only the apoprotein of the putative corrinoid protein Dhaf_4611 was formed. For protein purification and the incorporation of hydroxocobalamin or methylcobalamin as a corrinoid cofactor, a method described previously by Schilhabel et al. (31) was used.

Test for recombinant protein production using SDS-PAGE. At the times indicated, 1-ml samples were taken from *E. coli* cultures. After centrifugation (1 min at 16,000 × *g*), the cell pellets were stored at −21°C. For cell lysis, pellets were resuspended in 60 μl of 50 mM Tris HCl (pH 7.5) containing 0.2 mg/ml lysozyme and 0.02 mg/ml DNase I. After 1 h of incubation at 37°C at 1,000 rpm, cell debris was removed by centrifugation (5 min at 16,000 × *g*). The supernatant (soluble protein) was used for protein determination (see "Analytical methods" below). SDS-PAGE was performed according to a method described previously by Laemmli (20), using a Tris-glycine-SDS buffer system. Total protein was sampled directly after cell lysis.

Determination of enzyme activities. The enzyme activities of the putative *O*-demethylase components were determined in a final volume of 100 μl in anaerobic quartz cuvettes according to methods described previously by Schilhabel et al. (31).

The assay mixture for the determination of Dhaf_4610 (MT I) activity contained 50 mM DTT, 2 mM ATP, 10 mM $MgCl_2$, 0.5 mM titanium(III) citrate, 5 mM the respective substrate, 50 μM Dhaf_4611 (CP), and AE of *A. dehalogenans* (10 μg protein of recombinant *E. coli* crude extract). The reaction was started by the addition of Dhaf_4610 to the mixture. The enzyme activity was determined by measuring the formation of methylcobalamin from cob(I)alamin at 528 nm (ε_{528} = 7.9 mM^{-1} cm^{-1} [9]).

The assay mixture for the determination of Dhaf_4612 (MT II) activity contained 15 μM Dhaf_4611 (CP) reconstituted with methylcobalamin, 5 mM FH_4, and 0.5 mM titanium(III) citrate. The reaction was started by the addition of Dhaf_4612 to the mixture. The MT II activity was measured by monitoring the demethylation of methylated Dhaf_4611 at 528 nm (ε_{528} = 7.9 mM^{-1} cm^{-1} [9]).

The assay mixture for the determination of AE activity contained 2

TABLE 1 Oligonucleotides used for cloning of the putative *O*-demethylase genes of *Desulfitobacterium hafniense* DCB-2 into pET11a[a]

Gene	Primer sequence	PCR step
Dhaf_1265	CGC GTT CAT ATG AAT CAT TAT CGG CC	1
	CTG CGG GTG GCT CCA AGC GCT GCA GAG ACC CCT TTG	
	CGC GTT CAT ATG AAT CAT TAT CGG CC	2
	CAG CCG GAT CCT TAT TTT TCG AAC TGC GGG TGG C	
Dhaf_2573	CGC GTT CAT ATG GGT AAA GAA ATA AC	1
	CTG CGG GTG GCT CCA AGC GCT TGT TAT ACC TCC TTT TGG	
	CGC GTT CAT ATG GGT AAA GAA ATA AC	2
	CAG CCG GAT CCT TAT TTT TCG AAC TGC GGG TGG C	
Dhaf_2795	CGC GTT CAT ATG GAG AAA TAC CAG GTT AAA TTT ATG C	1
	CTG CGG GTG GCT CCA AGC GCT AAA TGT TCC TTC TAC AGA AG	
	CGC GTT CAT ATG GAG AAA TAC CAG GTT AAA TTT ATG C	2
	CAG CCG GAT CCT TAT TTT TCG AAC TGC GGG TGG C	
Dhaf_3310	CGC GTT CAT ATG GCT CAA GTT ACG	1
	CTG CGG GTG GCT CCA AGC GCT CTT TTC AAA TGA C	
	CGC GTT CAT ATG GCT CAA GTT ACG	2
	CAG CCG GAT CCT TAT TTT TCG AAC TGC GGG TGG C	
Dhaf_3879	CGC GTT CAT ATG AAA GAA GTC AGG ATA G	1
	CTG CGG GTG GCT CCA AGC GCT ATC CAA CTT AAA ATA G	
	CGC GTT CAT ATG AAA GAA GTC AGG ATA G	2
	CAG CCG GAT CCT TAT TTT TCG AAC TGC GGG TGG C	
Dhaf_4322	CGC GTT CAT ATG ATC ATC AGG ATA AAG CAT TAC	1
	CTG CGG GTG GCT CCA AGC GCT GTC TGT TGG CTC CAA ATA C	
	CGC GTT CAT ATG ATC ATC AGG ATA AAG CAT TAC	2
	CAG CCA GAT CTT TAT TTT TCG AAC TGC GGG TGG C	
Dhaf_4610	GGA GAT ATA CAT ATG TTG ACA ATT AAA CAA AAC TTG TTG G	1
	CTG CGG GTG GCT CCA AGC GCT GAA CAT TTT CTT GCT CAG	
	GGA GAT ATA CAT ATG TTG ACA ATT AAA CAA AAC TTG TTG G	2
	CAG CCG GAT CCT TAT TTT TCG AAC TGC GGG TGG C	
Dhaf_4611	GGA GAT ATA CAT ATG TCT AAA ATC GCA G	1
	CTG CGG GTG GCT CCA AGC GCT ATC ATG AGC CAA TTC TTT GGC T	
	GGA GAT ATA CAT ATG TCT AAA ATC GCA G	2
	CAG CCG GAT CCT TAT TTT TCG AAC TGC GGG TGG C	
Dhaf_4612	CGC GTT CAT ATG ATA CTC ATT GGT G	1
	CTG CGG GTG GCT CCA AGC GCT TTT CTG TCC GAA TTT AC	
	CGC GTT CAT ATG ATA CTC ATT GGT G	2
	CAG CCG GAT CCT TAT TTT TCG AAC TGC GGG TGG C	

[a] For details, see Materials and Methods.

TABLE 2 Plasmids and conditions used for expression of the putative *O*-demethylase genes of *Desulfitobacterium hafniense* DCB-2 in *E. coli* BL21(DE3)

Plasmid[a]	Inducing condition		
	Temp (°C)	IPTG (mM)	Time (h)
pET11a_Dhaf_4610*Strep*	28	0.25	4
pET11a_Dhaf_4611*Strep*	28	0.25	4
pET11a_Dhaf_4612*Strep*	18	0.25	4
pET11a_Dhaf_1265*Strep*	18	0.25	16
pET11a_Dhaf_2573*Strep*	37	0.25	4
pET11a_Dhaf_2795*Strep*	18	0.50	16
pET11a_Dhaf_3310*Strep*	18	0.50	16
pET11a_Dhaf_3879*Strep*	18	0.25	4
pET11a_Dhaf_4322*Strep*	28	0.25	4

[a] The NdeI and BamHI restriction sites of pET11a were used for the cloning of the target genes.

mM ATP, 10 mM $MgCl_2$, 0.5 mM titanium(III) citrate, and 30 μM Dhaf_4611 (CP). The reaction was started by the addition of the different recombinant COG3894 proteins of *D. hafniense* DCB-2 to the mixture. The activity of AE was calculated by measuring the formation of cob(I)alamin from cob(II)alamin at 386 nm ($\Delta\varepsilon_{386} = 21\ mM^{-1}\ cm^{-1}$ [31]).

Analytical methods. Protein determinations were performed according to the method of Bradford (4). Bovine serum albumin was used as the standard. The zinc content was determined by atomic absorption spectroscopy.

Bioinformatic analyses. The analysis of the genome of *D. hafniense* DCB-2 (GenBank accession no. CP001336.1) was performed with the Integrated Microbial Genomes (IMG) system supported by the Department of Energy Joint Genome Institute (22) as well as with the NCBI BLAST Server (1). The amino acid sequences of OdmA (CP) (GenBank accession no. AAC83695.2), OdmC (AE) (GenBank accession no. ACJ01666.1), and OdmD (MT II) (GenBank accession no. AAR11880.2)

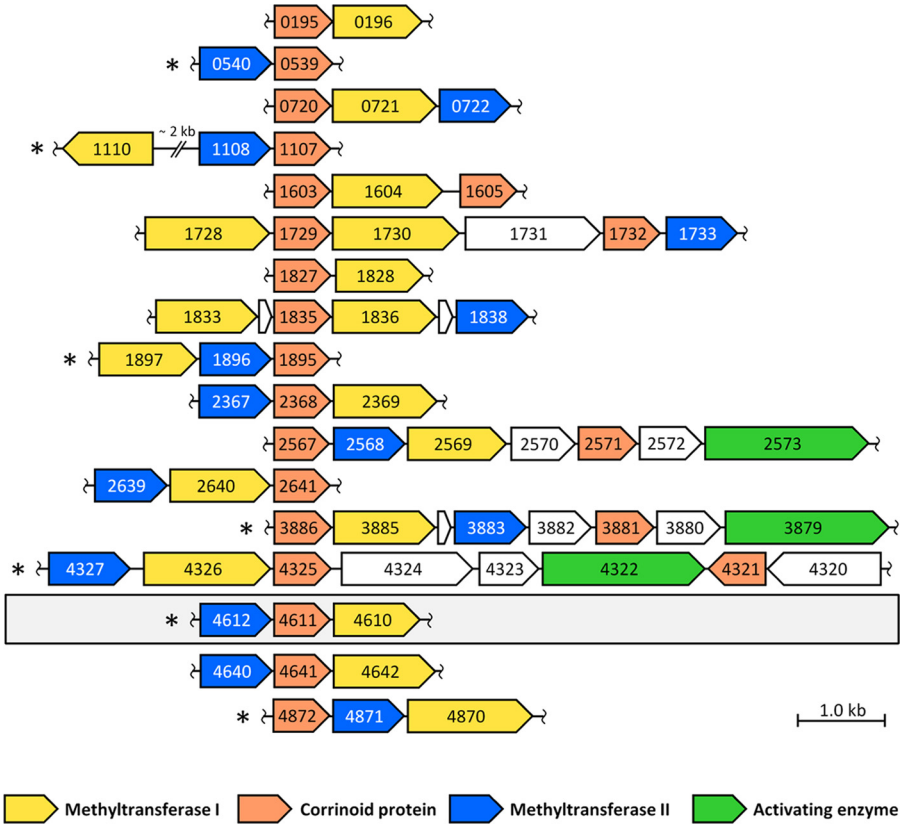

FIG 2 Putative *N*-, *S*- *Cl*-, or *O*-demethylase operons in the genome of *Desulfitobacterium hafniense* DCB-2 (GenBank accession no. CP001336.1). Gene clusters oriented in the reverse direction in comparison to the orientation in the annotated genome are marked with asterisks. In this study, the genes of the Dhaf_4610-Dhaf_4611-Dhaf_4612 operon (light gray box) were used for heterologous expression.

of the vanillate *O*-demethylase of *Acetobacterium dehalogenans* were used for BLASTP searches.

RESULTS

Bioinformatic analysis of *Desulfitobacterium hafniense* DCB-2 genes putatively encoding corrinoid-dependent methyltransferase systems. To identify putative *O*-demethylase genes in the genome of *D. hafniense* DCB-2, the highly conserved amino acid sequences of the corrinoid protein (CP) and methyltransferase II (MT II) of *Acetobacterium dehalogenans* were used for BLASTP searches. This screening was based on the following gene assignments: putative methyltransferase I (MT I) genes belong to the uroporphyrinogen-III decarboxylase family, putative MT II genes belong to the dihydropteroate synthase family, and CP genes are annotated as cobalamin binding proteins. Using this method, besides *O*-demethylases, *N*-, *S*-, and *Cl*-demethylase systems would also have been detected. The screening resulted in the identification of at least 17 putative operons encoding corrinoid-dependent methyltransferase systems (Fig. 2). All identified methyltransferase operons of *D. hafniense* DCB-2 consist of at least two of these three genes encoding MT I, MT II, and/or CP. In most cases, all three genes were present. The sequence identity values for CP or MT II at the protein level vary from 30 to 75% for *D. hafniense* DCB-2. For *A. dehalogenans*, the CPs known (5 proteins) exhibit identity values of 80 to 90% (our unpublished data); for MT II (3 known), the identity is 80 to 99%. MT I is responsible for the substrate specificity of the *O*-demethylase systems (19). Therefore, the low identity values of <30% for the different MT I proteins of *D. hafniense* DCB-2 were not surprising, since the identity of the characterized vanillate MT I (OdmB) and veratrole MT I (VdmB) of *A. dehalogenans* was also low (23%) (similarity, ~40%). Of the putative MT I genes listed in Fig. 2, the Dhaf_1728, Dhaf_1730, Dhaf_4326, and Dhaf_4870 genes exhibit more similarity to *N*- than to *O*-demethylase MT I genes. It cannot be excluded that other putative methyltransferase operons shown in Fig. 2 transfer *N*-, *S*- or *Cl*-bound methyl groups to corrinoid proteins.

Heterologous expression of the putative *O*-demethylase genes Dhaf_4610, Dhaf_4611, and Dhaf_4612. Two-dimensional (2D) gel electrophoresis of pyruvate-fumarate- and vanillate-fumarate-grown cells led to the identification of a putative CP obviously induced on cells cultivated with vanillate-fumarate. CP was identified as the Dhaf_4611 protein by the sequencing of peptides obtained by tryptic digestion (data not shown). In addition, a BLASTP search using the amino acid sequence of OdmB of *A. dehalogenans* (MT I of the vanillate *O*-demethylase [MT I_{van}]) revealed the highest similarity with the Dhaf_4610 protein. For these reasons, the genes of the putative *O*-demethylase operon encoding the components Dhaf_4610 (MT I), Dhaf_4611 (CP), and Dhaf_4612 (MT II) were chosen for heterologous expression in *Escherichia coli*. The genes were separately cloned as Strep tag fusions into the pET11a expression vector (see Materials and Methods). To obtain large amounts of soluble recombinant pro-

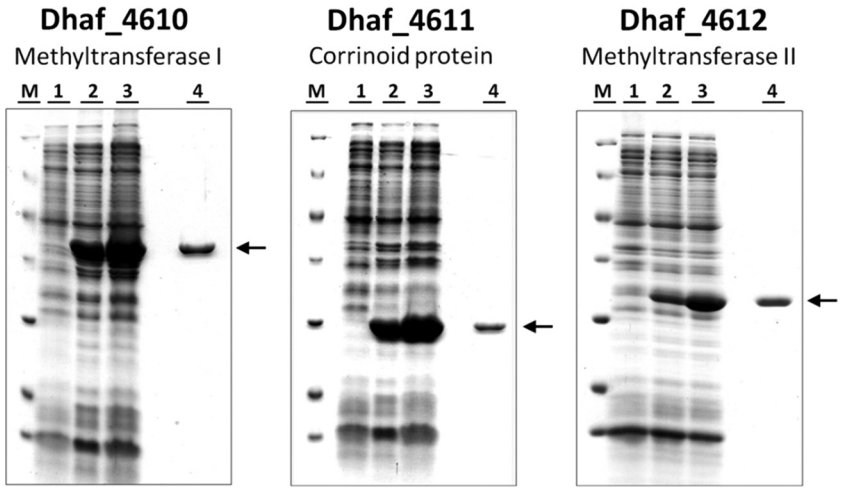

FIG 3 SDS-PAGE of heterologously expressed Dhaf_4610, Dhaf_4611, and Dhaf_4612 proteins. The proteins were stained with Coomassie blue. The apparent molecular masses of the marker proteins (lanes M) are 116, 67, 45, 35, 25, 18, and 14 kDa, respectively. For each component, 5 μg of crude extract before (lanes 1), 2 h after (lanes 2), and 4 h after (lanes 3) induction with IPTG and 1 μg of purified protein (lanes 4) were loaded.

tein (Fig. 3, lanes 2 and 3), the conditions for heterologous expression were optimized (Table 2). By affinity chromatography on *Strep*-Tactin, the recombinant methyltransferases were purified to apparent homogeneity in a single purification step (Fig. 3, lane 4). Dhaf_4611 (CP) was purified by using anion-exchange chromatography according to methods described previously by Schilhabel et al. (31), since the reconstitution of the cofactor-free recombinant apoprotein with cobalamin was performed during the course of this procedure. The apparent molecular masses determined via SDS-PAGE were indistinguishable from the molecular masses calculated from the respective amino acid sequences.

Characterization of the recombinant proteins Dhaf_4610, Dhaf_4611, and Dhaf_4612. Dhaf_4610 encodes a putative MT I, Dhaf_4611 encodes a putative CP, and Dhaf_4612 encodes a putative MT II. MT I catalyzes the cleavage of the substrate ether bond and the transfer of the methyl group to the superreduced corrinoid cofactor of CP. MT II is responsible for the transfer of the methyl group from CP to FH_4, yielding methyl-FH_4. Thus, a prerequisite for all enzyme measurements is the availability of a functional CP. Dhaf_4611 was enriched by using anion-exchange chromatography, and either hydroxocobalamin or methylcobalamin was incorporated as the corrinoid cofactor. When hydroxocobalamin was used, a fraction containing a reddish-brown protein solution was obtained from the last purification step (chromatography on Mono Q). This indicated the cob(II)alamin form of CP ($[Co^{II}]$). To prove the functionality of Dhaf_4611, a corrinoid reduction assay with recombinant AE of *A. dehalogenans* was performed (31). In this assay, the corrinoid cofactor of Dhaf_4611 was reduced in an ATP-dependent reaction from cob(II)alamin to cob(I)alamin, indicating a functional CP. For the determination of MT I activity, hydroxocobalamin-containing Dhaf_4611 and AE of *A. dehalogenans* were used in the assays.

The activity of Dhaf_4610, a putative MT I, was tested with different phenyl methyl ethers as the substrate and compared to the substrate spectrum of the vanillate *O*-demethylase (Odm) and veratrole *O*-demethylase (Vdm) of *A. dehalogenans* (Table 3). The formation of methylated CP (CH_3-$[Co^{III}]$) from the superreduced CP ($[Co^{I}]$) and the substrate (CH_3-X) was measured according to the following equation: $CH_3\text{-X} + [Co^{I}] \rightarrow X + CH_3\text{-}[Co^{III}]$ (MT I).

The decrease of the absorption of $[Co^{I}]$ at 386 nm and the increase of the absorption of methyl-CP at 528 nm were monitored photometrically (shown for vanillate as the substrate in Fig. 4A). The reaction was started by the addition of Dhaf_4610 to the mixture. The formation of methylcobalamin could be measured for vanillate, guaiacol, isovanillate, syringate, and methyl chloride, confirming the role of Dhaf_4610 as MT I (Table 3). The highest specific activity (~50 nkat/mg) was achieved with guaiacol as the substrate. No MT I activity was observed with 3,4,5-trimethoxybenzoate, 3-hydroxyanisole, veratrole (Table 3), dichloromethane, methylamine, or methanol (data not shown). For all converted substrates, the specific activities of Dhaf_4610 were higher than those reported for the two *A. dehalogenans* methyltransferases (Table 3). The substrate spectrum of MT I_{van} of *A. dehalogenans* was similar to that of Dhaf_4610, except that the activity of the latter enzyme with guaiacol was very high and that methyl chloride served as a substrate for Dhaf_4610 rather than for MT I_{van}. The apparent K_m value of Dhaf_4610 for guaiacol was about 60 μM, and that for vanillate was about 5 mM. The activities given in Table 3 were measured for 5 mM concentrations of the phenyl methyl ethers. The extrapolated V_{max} value for vanillate was near 26 nkat/mg.

Different corrinoid-dependent methyltransferases, such as the MTs I of *A. dehalogenans*, the methanol methyltransferase MtaB of *Methanosarcina barkeri*, and the methionine synthase of *E. coli*, were previously described to be zinc-containing proteins (10, 27, 31). Zinc determinations of Dhaf_4610 revealed a substoichiometric amount of approximately 0.2 mol zinc per mol MT I. An incubation of the protein with additional zinc did not result in an increase of the zinc content of Dhaf_4610. In addition, zinc had no influence on the enzyme activity when directly added to the assay mixture (data not shown). The addition of the metal-chelating agent EDTA (final concentration, 1 mM) led to a minor decrease of the enzyme activity (77% remaining activity). In the presence of 5 mM EDTA, the enzyme was still active (60% remaining activity).

TABLE 3 Specific activities and substrate spectrum of the methyltransferase I Dhaf_4610 in comparison to the activities of the recombinant methyltransferases I (rMT I_{van} and rMT I_{ver}) of the vanillate and veratrole *O*-demethylases of *A. dehalogenans*[a]

Substrate	Sp act (nkat/mg)		
	Dhaf_4610	rMT I_{van}	rMT I_{ver}
Vanillate	13.28	2.43	0.32
Isovanillate	16.97	5.82	2.09
Syringate	8.44	2.43	1.05
3,4,5-Trimethoxybenzoate	<0.05	<0.05	<0.05
Guaiacol	50.21	1.26	1.52
3-Hydroxyanisole	<0.05	<0.05	3.93
Veratrole	<0.05	<0.05	3.29
Methyl chloride	39.88	<0.05	0.44

[a] See reference 31.

Under these conditions, the AE activity, which was necessary for a functional assay, was not inhibited by EDTA.

Dhaf_4612, as a putative MT II, was expected to catalyze the transfer of the methyl group from CP-bound methylcobalamin to tetrahydrofolate (FH_4), yielding methyl-FH_4 and CP-bound cob(I)alamin. Due to its low redox potential (32), the cob(I)alamin cofactor of CP is immediately converted to cob(II)alamin *in vitro* when AE is absent. The assay mixture used did not contain AE. Therefore, the conversion of the methylcobalamin cofactor of CP to cob(II)alamin was measured. The assay mixture contained FH_4 and methylated CP obtained by the reconstitution of the Dhaf_4611 apoprotein with methylcobalamin as the corrinoid cofactor. The reaction was strictly dependent on the presence of tetrahydrofolate and was measured as follows: CH_3-[Co^{III}] + $FH_4 \rightarrow CH_3$-FH_4 + [Co^{I}] (MT II) and [Co^{I}] $\rightarrow$ [Co^{II}] + e^- (inadvertent oxidation).

The reaction was started by the addition of Dhaf_4612 to the mixture. Subsequently, after the addition, the absorption at 528 nm (absorption maximum of methylcobalamin) decreased, and a peak at 475 nm, indicating the formation of cob(II)alamin, appeared (Fig. 4B). This reaction corroborated the putative function of Dhaf_4612 as MT II. The specific activity was about 150 nkat/mg. This activity was 2 orders of magnitude higher than the activity of the purified recombinant MTs II of *A. dehalogenans* (our

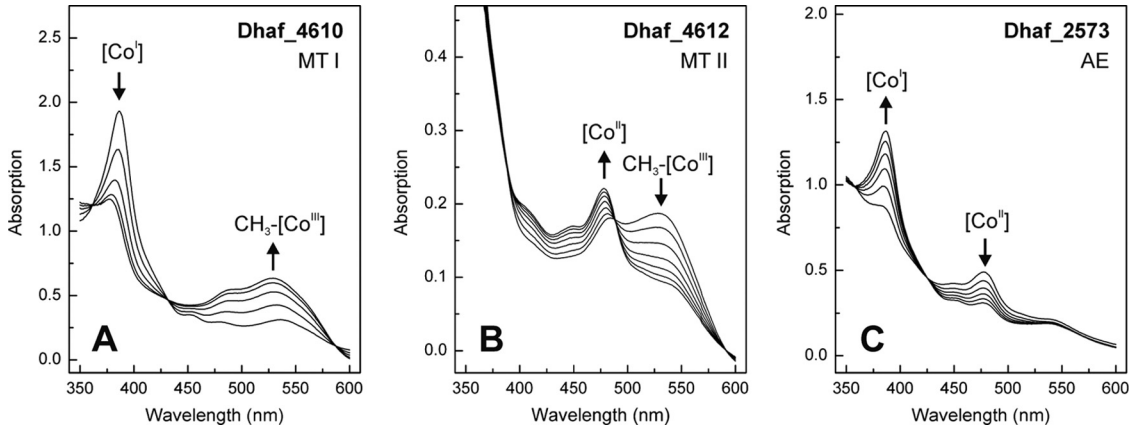

FIG 4 Absorption spectra of the corrinoid cofactor of Dhaf_4611 (CP) during measurement of Dhaf_4610 (MT I) (A), Dhaf_4612 (MT II) (B), and Dhaf_2573 (AE) (C) activities. A spectrum was recorded every 30 s. In the assay mixtures, the final concentrations of Dhaf_4610, Dhaf_4612, and Dhaf_2573 were 13 μM, 0.5 μM, and 16 μM, respectively. The assay reactions are described in the text. For details, see Materials and Methods.

unpublished data). Zinc could not be detected in the purified enzyme (<0.1 mol per mol protein).

Putative AEs of *Desulfitobacterium hafniense* DCB-2. The AE of *A. dehalogenans* is a member of the COG3894 protein family. Therefore, a BLASTP search for COG3894 proteins was performed. This screening revealed six COG3894 proteins encoded in the genome of *D. hafniense* DCB-2 (Dhaf_1265, Dhaf_2573, Dhaf_2795, Dhaf_3310, Dhaf_3897, and Dhaf_4322). With the exception of Dhaf_1265, all proteins contain a putative [2Fe-2S] cluster binding motif in the N-terminal region. Three of the Fe/S-containing COG3894 protein-encoding genes were located close to putative *O*-demethylase operons (Fig. 2). To study the physiological role of the different COG3894 proteins of *D. hafniense* DCB-2, the corresponding genes were cloned separately as Strep tag fusions into pET11a, and the conditions for recombinant protein production in *E. coli* BL21(DE3) were optimized (Table 2). The formation of the different COG3894 proteins after induction is shown in Fig. 5. After the addition of IPTG, all proteins were produced (Fig. 5, lanes 2, total protein). Using lysozyme treatment, cell lysis did not yield soluble Dhaf_3310 (Fig. 5, lanes 3). Solubilization was achieved by cell disruption in a French pressure cell (Fig. 5, lanes 4). The recombinant proteins Dhaf_1265, Dhaf_2573, Dhaf_2795, Dhaf_3310, Dhaf_3897, and Dhaf_4322 were purified to apparent homogeneity by affinity chromatography on *Strep*-Tactin (Fig. 5, lanes 5). The apparent molecular masses determined via SDS-PAGE were similar to the molecular masses calculated from the respective amino acid sequences.

The recombinant COG3894 proteins of *D. hafniense* DCB-2 were tested for their function as an AE, i.e., the activation and reduction of CP-bound corrinoids, according to the following equation: $[Co^{II}] + e^- + ATP + H_2O \rightarrow [Co^{I}] + ADP + P_i$ (AE).

The assay was performed as described previously for *A. dehalogenans* (31). Recombinant Dhaf_4611 with hydroxocobalamin as the corrinoid cofactor ($[Co^{II}]$-form) was used as the CP, and titanium(III) citrate was used as the electron donor. The reaction was started by the addition of the putative AEs. The formation of cob(I)alamin from cob(II)alamin was measured by the absorption increase at 386 nm and the absorption decrease at 475 nm. As a positive control, the AE of *A. dehalogenans* was used. Dhaf_1265, Dhaf_2795, and Dhaf_3310 showed no activity. Dhaf_3879 and Dhaf_4322, the corresponding genes of which are located close to putative *O*-demethylase operons (Fig. 2), exhibited low activity (≤0.05 nkat/mg), which, however, was not significant in comparison to that of the *A. dehalogenans* AE (up to 10 nkat/mg). The gene encoding Dhaf_2573 is also located close to a putative *O*-demethylase operon (Fig. 2). Dhaf_2573 was the only COG3894 protein that exhibited significant AE activity (Fig. 4C); the activity was strictly dependent on the presence of ATP. The specific activity of this enzyme was calculated to be 0.5 nkat/mg. This activity is 1 order of magnitude lower than that of the recombinant *A. dehalogenans* AE after reconstitution with Fe and S (31). The Fe content of Dhaf_2573 was about 1.7 mol Fe per mol protein (data not shown), which is in accordance with the presence of a [2Fe-2S] cluster, which was to be expected from the amino acid sequence. This finding indicated that the low specific activity was probably not due to a loss of Fe during purification. Moreover, the activity could not be increased by reconstitution with Fe and S.

DISCUSSION

In this report, we describe the characterization of recombinant *O*-demethylase components of *Desulfitobacterium hafniense* strain DCB-2. These four-component enzyme systems mediate the cleavage of phenyl methyl ethers. The latter compounds, among other aromatics, are formed upon fungal lignin degradation. They can be demethylated under anoxic conditions by acetogenic bacteria, which use the methyl group of phenyl methyl ethers as the energy and carbon source (2, 13, 23). Recently, it was demonstrated that *Desulfitobacterium* is also able to utilize these compounds; however, growth with phenyl methyl ethers depends on the presence of an electron acceptor such as fumarate or chlorinated aromatics (26). Since phenyl methyl ethers in forest soil often are chlorinated, due to modification by fungal or bacterial haloperoxidases (11), it is feasible that the organism can utilize reductive dechlorination as an electron-accepting reaction for the oxidation of the methyl group of phenyl methyl ethers. Therefore, it is not surprising that *Desulfitobacterium hafniense* can be isolated from forest soil (our unpublished data). Hence, forest soil might be an appropriate inoculum for the enrichment of these bacteria, as shown previously for *Desulfitobacterium frappieri* PCP-1 (21).

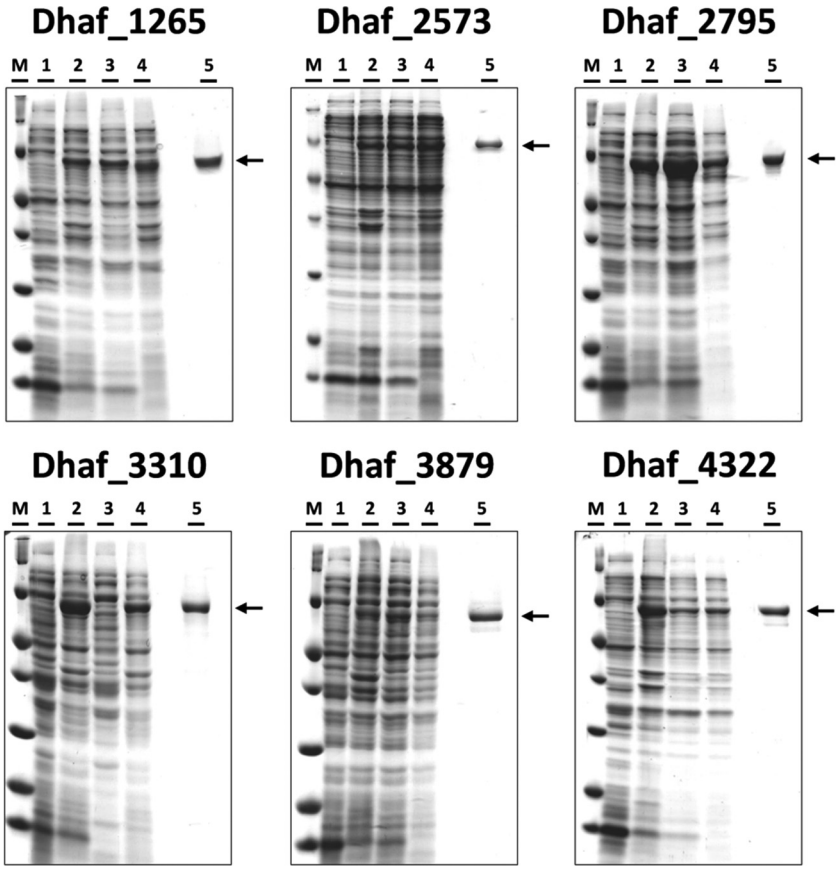

FIG 5 SDS-PAGE of heterologously expressed COG3894 proteins of *Desulfitobacterium hafniense* DCB-2. The proteins were stained with Coomassie blue. The apparent molecular masses of the marker proteins (lanes M) are 116, 67, 45, 35, 25, 18, and 14 kDa, respectively. For each component, 5 μg of crude extract before (lanes 1), and after (lanes 2, 3, and 4) induction with IPTG and 1 μg of purified protein (lanes 5) were loaded. Lanes 2, total protein; lanes 3, soluble protein after lysozyme treatment; lanes 4, soluble protein after French press disruption. For detailed information, see Materials and Methods.

In the genome of *D. hafniense* DCB-2, 17 putative operons were detected, which may encode corrinoid-dependent methyltransferase systems. In acetogens such as *Moorella thermoacetica*, numerous methyltransferase operons can also be found (28). This is not surprising, since upon lignin degradation, a variety of phenyl methyl ethers is formed. In *Acetobacterium dehalogenans*, different *O*-demethylases are induced by different aryl ethers (7; our unpublished data). Each of these enzyme systems can use different phenyl methyl ethers; the substrate spectra are sometimes even overlapping. This may also be the case for *Desulfitobacterium*. Here, we characterized one of these enzyme systems in detail. The enzyme also had a wide substrate spectrum, which was similar to that of the *A. dehalogenans* vanillate *O*-demethylase. Of the aryl ethers tested, only those with a hydroxyl substituent in the ortho position of the methoxy group were demethylated by methyltransferase I (MT I), as also reported previously for *A. dehalogenans* OdmB (15). Whereas MT I of the latter enzyme system converted vanillate and isovanillate at the highest rates (approximate K_m for vanillate of 100 μM and approximate K_m for guaiacol of 0.8 mM), the activity of *Desulfitobacterium* MT I (Dhaf_4610) was higher with guaiacol than with vanillate. The apparent K_m value of Dhaf_4610 for guaiacol (60 μM) was lower than that for vanillate (5 mM) by 2 orders of magnitude. This result indicates that under physiological conditions, i.e., at low phenyl methyl ether concentrations, guaiacol may be the preferred substrate for this *O*-demethylase.

A remarkable exception between the two MTs I was the conversion of methyl chloride by *D. hafniense* MT I; CH_3Cl was not a substrate for the *A. dehalogenans* enzyme. However, an MT I_{van} of the *A. dehalogenans* vanillate *O*-demethylase that was truncated at its N terminus was also able to convert methyl chloride (19). This indicates that only minor structural changes in the enzyme may lead to this difference in the substrate spectrum. An amino acid sequence identity of 66% (80% similarity) of the two MTs I corroborates the functional similarity of these enzymes. The conversion of methyl chloride by this methyltransferase explains why *D. hafniense* DCB-2 is able to grow with methyl chloride as the electron donor and fumarate as the electron acceptor (data not shown).

The corrinoid-dependent methyltransferases of anaerobic bacteria and archaea have been described to contain zinc as a cofactor (10, 27, 31). In the recombinant MT I of *D. hafniense* DCB-2, about 0.2 mol Zn per mol protein was detected. Either this enzyme is an exception to the rule, or, upon MT I production or purification, a major portion of the cofactor was lost. We cannot rule out the latter possibility, although this would imply that the already high specific activity of the holoprotein is 5 times higher than that measured for the recombinant enzyme. When the deduced amino

acid sequences of both MTs I were compared, further similarities could be detected. Structure predictions for both enzymes suggested a C-terminal TIM-barrel structure (where TIM is triose phosphate isomerase); this structure was proposed to play a major role in catalysis, whereas the N terminus was shown to be responsible for substrate binding (19). In MT I_{van} of *A. dehalogenans*, an unusual zinc binding motif was identified, which was localized in the N-terminal part of the TIM-barrel structure (33). This motif consists of three amino acids, E132, E147, and H168. The first two amino acids are found in the corresponding positions (E131 and E146) of the *Desulfitobacterium* enzyme, whereas the histidine residue is missing. It might, however, be replaced by a different zinc binding partner.

The corrinoid protein (CP) Dhaf_4611 was produced as a cofactor-free apoprotein due to the fact that *E. coli* is not able to synthesize vitamin B_{12}. The apoprotein could be reconstituted with hydroxocobalamin. It cannot be excluded that *in vivo*, the cofactor is a different B_{12} derivative, since the organism contained different species of corrinoids (our unpublished data). Nonetheless, the reconstituted CP was active in the corrinoid reduction and methyltransferase assays. It is feasible, however, that the activities with the native CP would be higher than those measured. The corrinoid of the CP (Dhaf_4611) was reduced by the activating enzyme (AE) and methylated by MT I_{van} of *A. dehalogenans* in the presence of vanillate (data not shown), indicating a high functional similarity of the CPs of both organisms. This assumption is supported by the 73% identity (similarity, 86%) of CP_{van} of *A. dehalogenans* with Dhaf_4611. Dhaf_4611, when reconstituted with methylcobalamin, could serve as the methyl donor for the methylation of tetrahydrofolate in the MT II reaction. This was measured with Dhaf_4612 as the catalyst. The deduced amino acid sequence of Dhaf_4612 was about 70% identical (similarity, ~85%) to different MTs II of *A. dehalogenans* (GenBank accession no. AAR11880.2 and AAQ89564.1).

Six genes encoding COG3894 proteins were identified in the genome of *D. hafniense* strain DCB-2. These proteins were proposed previously to reduce the corrinoid cofactors of corrinoid proteins of methyltransferases after inadvertent oxidation to cob(II)alamin (8, 31). Therefore, they were referred to as RACE (reductive activator of corrinoid-dependent enzymes). The six COG3894 proteins of *D. hafniense* DCB-2 were produced in *Escherichia coli*, and the activity of the reduction of the CP (Dhaf_4611) was measured with the *E. coli* crude extracts. Significant activity was observed only with Dhaf_2573, which was purified and further characterized. The specific activity of the purified protein (~0.5 nkat/mg) was lower than that of the AE of *A. dehalogenans* by 1 order of magnitude. It cannot be excluded that this low activity was due to the possibly wrong cofactor of the reconstituted Dhaf_4611 (see above); however, since this protein has a "repair" function that is probably required only occasionally in the course of the methyl transfer reaction cycle (Fig. 1), the activity may be sufficient for sustaining the ether cleavage. Compared to the specific activity of the purified methanogenic RACE RamA (about 10^{-3} nkat/mg), involved in the methyl transfer from methylamine to coenzyme M, the activity of Dhaf_2573 was actually high. Dhaf_3879 and Dhaf_4322 also showed some activity, which was only about 1 to 10% of the Dhaf_2573 activity. The other three COG3894 proteins were not active. They may serve as RACE in other corrinoid-dependent reactions.

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