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MecE, MecB, MecC proteins orchestrate methyl group transfer during dichloromethane fermentation

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Running head: *Dehalobacterium* methyltransferases

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Abstract

Dichloromethane (DCM), a common hazardous industrial chemical, is anaerobically metabolized by four bacterial genera: *Dehalobacter*, *Dehalobacterium*, *Ca*. Dichloromethanomonas, *Ca*. Formimonas. However, the pivotal methyltransferases responsible for DCM transformation have remained elusive. In this study, we investigated the DCM catabolism of *Dehalobacterium formicoaceticum* strain EZ94, contained in an enriched culture, using a combination of biochemical approaches. Initially, enzymatic assays were conducted with cell-free protein extracts, after protein separation by blue native polyacrylamide gel electrophoresis. In the slices with the highest DCM transformation activity a high absolute abundance of the methyltransferase MecC was revealed by mass spectrometry. Enzymatic activity assays with heterologously expressed MecB, MecC, and MecE from strain EZ94 showed complete DCM transformation only when all three enzymes were present. Our experimental results, coupled with the computational analysis of MecB, MecC, and MecE sequences enabled us to assign specific roles in DCM transformation to each of the proteins. Our findings reveal that both MecE and MecC are zinc-dependent methyltransferases responsible for DCM demethylation and re-methylation of a product, respectively. MecB functions as a cobalamin-dependent shuttle protein transferring the methyl group between MecE and MecC. This study provides the first biochemical evidence of the enzymes involved in the anaerobic metabolism of DCM.

Importance

Dichloromethane (DCM) is a priority regulated pollutant frequently detected in groundwater. In this work, we identify the proteins responsible for the transformation of DCM fermentation in *Dehalobacterium formicoaceticum* strain EZ94 using a combination of biochemical approaches, heterologous expression of proteins and computational analysis. These findings provide the basis to apply these proteins as biological markers to monitor bioremediation processes in the field.

Keywords: *In vitro* activity test, anaerobic fermentation, heterologous expression, methyltransferase, metalloprotein, *Dehalobacterium*.

INTRODUCTION

Dichloromethane (DCM), also known as methylene chloride, is a ubiquitous compound produced by both, natural sources, such as oceanic emissions, biomass burning, and volcanoes (1, 2), and anthropogenically through its widespread use as a solvent and intermediate in the chemical industry. DCM often becomes a groundwater contaminant due to improper disposal or spills (3-5). Once leaked, DCM, which is denser than water, can percolate through the soil and accumulate at the bottom of the aquifer as a dense non-aqueous liquid phase. Additionally, microbially mediated processes, including the reductive dechlorination of trichloromethane (TCM), can also contribute to DCM occurrence (6,7).

Biodegradation of DCM under oxic and anoxic conditions has been repeatedly reported. Most DCM-degrading bacteria described in the literature have been isolated from oxic environments. A representative example are facultative methylotrophs catalyzing DCM transformation, *e.g.*, through DcmA, a glutathione-*S*-transferase (GST) that leads to formaldehyde (8,9). However, as oxygen is often limited in contaminated aquifers, anaerobic bacteria become viable candidates for groundwater bioremediation. Under nitrate-reducing and methanogenic conditions, anaerobic microbial transformation of DCM has been reported (10,11). To date, four anaerobic bacterial species capable of transforming DCM have been reported: *Dehalobacterium formicoaceticum* strains DMC and EZ94, that ferment DCM into formate, acetate and inorganic chloride (12-16), *Candidatus* Dichloromethanomonas elyunquensis that mineralizes DCM into H_2, CO_2 and inorganic chloride (17,18), *Ca.* Formimonas warabiya, formerly referred to as strain DCMF, that ferments DCM into acetate and inorganic chloride (19), and a *Dehalobacter* sp. contained in the mixed cultures DCME and SC05-UT (derived from KB-1 Plus CF) that mineralizes DCM into H_2, CO_2 and inorganic chloride (20). *Ca.* Formimonas warabiya is the only bacterium described to use other non-chlorinated substrates such as methanol, choline, and glycine betaine (quaternary amines) besides DCM (19,21), while *D. formicoaceticum* strain DMC is the only isolate.

Recently, all the genomes of these bacteria or metagenomes from enriched cultures with them were sequenced and annotated. The absence of reductive dehalogenase genes in the genomes of *D. formicoaceticum* and *Ca.* Formimonas warabiya, coupled with the abundance of methyltransferase genes, led to the hypothesis that methyltransferases might play a pivotal role in the initial step of DCM transformation (12,22). In contrast, *Ca*. Dichloromethanomonas elyunquensis, despite encoding numerous methyltransferases, also encodes three reductive dehalogenases (*rdhA*) in its genome, expressing two of them in cultures growing with DCM (19). In the metagenome of the mixed culture containing *Dehalobacter* SC05-UT and DCME, only one reductive dehalogenase (AcdA) was detected in the proteome during complete TCM transformation, although 27 *rdhA* genes are encoded in its genome. In this case, the authors argued that AcdA cannot dechlorinate DCM but TCM, asserting that methyltransferases might be the primary enzymes responsible for DCM transformation in *Dehalobacter* (20). Consequently, methyltransferases were pinpointed as the primary agents responsible for DCM dechlorination, although the role of reductive dehalogenases in the genus *Ca*. Dichloromethanomonas elyunquensis remains unexplained.

In the late 1990s, physiological and biochemical investigations on *D. formicoaceticum* strain DMC using crude cell extracts revealed that DCM combined with tetrahydrofolate (THF) produces 5,10-methylene-THF, which subsequently enters the Wood-Ljungdahl pathway (15). Additionally, these studies postulated that the transformation of DCM was corrinoid-dependent, although the catalytic enzyme was not identified (15). At present, it is widely accepted that the methyl group of the DCM is metabolized by the Wood-Ljungdahl pathway in all reported anaerobic DCM degraders, even though different catabolic pathways for DCM might operate among different genera.

Proteogenomic studies on *D. formicoaceticum* and *Ca*. Dichloromethanomonas elyunquensis have identified a methyltransferase cassette, denominated the *mec* (methylene chloride catabolism) cassette. The cassette, comprising ten genes from *mecA* through *mecJ*, is suspected to be implicated in DCM transformation (23). Cultures of *D. formicoaceticum*, *Ca*. Dichloromethanomonas

elyunquensis and *Dehalobacter* sp. contained in the mixed cultures DCME and SC05-UT produced a large number of proteins from the *mec* cassette, among them putative methyltransferases, during growth with DCM (20,23). Moreover, *mecE* and *mecF* gene transcript levels in DCM-contaminated groundwater correlated well with DCM pollution (23). Despite these findings, the key enzyme for DCM transformation remains unknown in the four mentioned bacteria.

In our previous work, we have enriched an anaerobic DCM-fermenting bacterial consortium from a membrane bioreactor at an industrial wastewater treatment plant (24). This consortium was dominated by the genera *Dehalobacterium*, *Acetobacterium*, *Desulfovibrio*, and *Wolinella*, and produced acetate, formate and chloride when grown with DCM in a bicarbonate-buffered medium (16,24). Recently, we obtained the assembled genome of a *Dehalobacterium* strain in the consortium, designated *Dehalobacterium formicoaceticum* strain EZ94, through metagenomic sequencing and used the genome information to characterize the expressed proteome during growth with DCM (25).

The objective of the current study was to identify the enzymes involved in the DCM fermentation process using *D. formicoaceticum* strain EZ94 as model organism. To achieve this, we employed various approaches, including enzymatic assays using cell-free crude extracts separated through blue native polyacrylamide gel electrophoresis (BNE), and protein mass spectrometry. Additionally, we heterologously expressed candidate genes in *Escherichia coli* BL21 (DE3) and conducted enzyme activity assays with the recombinant methyltransferases. These approaches enabled us to pinpoint MecB, MecC, and MecE, all encoded in the *mec* cassette, to be involved in DCM transformation. Bioinformatic analyses, including *in-silico* protein structure predictions of MecB, MecC, and MecE, multiple sequence alignments, and the prediction of cofactor binding sites, enabled us to assign specific functions in the transformation of DCM to the three proteins.

RESULTS

Abundance and functional predictions of proteins from the *mec* operon in *D. formicoaceticum* strain EZ94

First, we analyzed the proteome profile of the crude extract and the soluble protein fraction of the *Dehalobacterium*-containing culture growing with DCM using shotgun proteomics. As recently reported (23), the methyltransferases from the *mec* operon (Supplementary Figure 1) were among the most abundant proteins (Supplementary Table 1). MecC was the most abundant protein in both the protein crude extract and the soluble protein fraction. MecE, MecB, and MecF were also among the top ten most abundant proteins in both samples (Supplementary Table 1). MecB is annotated as a corrinoid methyltransferase, MecC and MecE are predicted as methylcobalamin methyltransferases, whereas MecF is anticipated to act as a tetrahydromethanopterin methyltransferase. MecI was identified in the soluble protein fraction and in the protein crude extract, ranking at a relative abundance position of 122 and 110, respectively (comparing the absolute abundance values of MS1 peak intensities within one sample), while MecH and MecJ were detected only in the protein crude extract ranking at relative abundance positions of 377 and 592, respectively (Supplementary Table 1). Functional annotation suggests that MecI serves as a uroporphyrinogen decarboxylase, MecH as a methyltransferase corrinoid activation protein, and MecJ as a Na^+/H^+ antiporter. The remaining proteins of the Mec cassette, namely MecA, MecD, and MecG, were not detected in either the crude protein extract or the soluble protein fraction. These proteins are predicted to function as regulatory proteins, which might explain the lack of detection.

Dichloromethane transformation activity in protein fractions of *D. formicoaceticum* strain EZ94

To localize the DCM transforming activity, enzymatic activity assays were conducted using different protein fractions of strain EZ94 from the enriched culture: crude protein extract, membrane protein

fraction, and soluble protein fraction. Dechlorination/demethylation activity against DCM was detected in both the protein crude extract and the soluble protein fraction, while it was absent in the membrane protein fraction (Supplementary Figure 2). Subsequently, the soluble fraction was fractionated by size into three distinct mass fractions using ultrafiltration with defined exclusion sizes: *(i)* a soluble fraction below 30 kDa, *(ii)* a fraction of 30–100 kDa, and *(iii)* a fraction above 100 kDa. Transformation of DCM was solely observed in the soluble protein fraction above 100 kDa (Supplementary Figure 2). Henceforth, the soluble protein fraction containing proteins and protein complexes exceeding 100 kDa will be referred to as "large protein fraction" throughout this study. The transformation of DCM was inhibited in the presence of propyl iodide, a compound known to specifically target cobalamin-dependent proteins (26), suggesting the potential involvement of a cobalamin-dependent methyltransferase in DCM transformation.

The large protein fraction was fractionated using anion exchange chromatography into 17 fractions (see the section in Supplementary Results). Among these, the three fractions exhibiting DCM transformation activity displayed high absorbance at 360 nm, indicative of cob(III)alamin (Supplementary Figure 3). Subsequent mass spectrometric analysis of the proteins within these fractions identified the presence of methyltransferases MecB, MecC, MecE, and MecF, indicating their involvement in the observed activity.

Identification of putative Mec proteins involved in dichloromethane transformation using BNE followed by enzymatic assays and protein mass spectrometry

To investigate the function of the Mec proteins in DCM transformation, BNE was conducted with the large protein fraction from the *Dehalobacterium*-containing mixed culture. BNE separates proteins under non-denaturing conditions, enabling us to investigate DCM transformation activity in gel slices after protein separation and 24 h of incubation with reaction mixture.

In the initial experiment, gel lanes were loaded with protein from the large protein fraction, and each replicate lane was cut into 6 slices. The highest DCM transformation activity was observed in slices 3 (54.1 ± 43.4% DCM transformation after 24 h of incubation) and 4 (44.8 ± 10.1%), corresponding to the molecular masses of 68–166 kDa and 166–322 kDa, respectively (Supplementary Figure 4A–D). Subsequently, a second BNE was conducted with protein from the large protein fraction, and the gel lanes were cut into 12 slices. Complete transformation of DCM was observed in slice 8 in both replicate samples (Figure 1A, Supplementary Figure 4E), with the calculated molecular mass of the active slice in the range of 114–215 kDa (Figure 1A). Gel slices obtained from stained lanes of the BNE gel were excised, trypsin-digested, and analyzed by mass spectrometry. The absolute protein abundance and the relative distribution across the gel slices of proteins encoded in the *mec* operon were evaluated for both experiments (Figure 1B, Supplementary Figure 4F). In both BNE gel experiments, positive controls were included. They contained 0.7 and 1.5 mg protein of the large protein fraction, respectively, and exhibited DCM transformation rates of 91.0 ± 8.0 and 100 ± 0%, respectively. An additional positive control was the sample prepared for gel loading, which contained 1.4 and 2.2 mg of large protein fraction supplemented with 0.125% (w/v) Coomassie G-250, which were not loaded onto the BNE. These samples showed 100% DCM transformation activity. Negative controls lacking enzymes did not show DCM transformation after 24 h of incubation (data not shown).

The nLC-MS/MS analysis revealed that in both BNE analyses, MecC was the predominant protein in the active slices, which absolute protein abundance (MS1 peak intensity approximately 3×10^9) was expressed one order of magnitude higher than other Mec proteins such as MecF, MecB, MecE (MS1 peak intensities between 1.0×10^8 and 5.0×10^8), and two orders of magnitude higher than MecI and MecH (Supplementary Figure 5). In the BNE gel cut into 12 slices, MecF co-eluted with MecC, showing comparable relative abundance in slices 8 and 9 (Figure 1). However, complete

DCM transformation was only detected in slice 8, where MecC exceeded MecF in relative and absolute abundance. Similar observations were made for the BNE gel lane cut in 6 slices, where MecC, in absolute terms, was the most abundant protein identified in the active gel slices 3 and 4 (Supplementary Figure 4B, 4D and 5A). In both gels, MecB and MecE were found in the same gel slices representing a gel area for proteins with molecular mass below 50 kDa. No DCM transformation was observed in these slices (slice 4 in Figure 1 and Supplementary Figure 4E, and slice 2 in Supplementary Figure 4A and 4C). Thus, both BNE gels display a similar pattern of Mec protein separation and activity distribution across the gel, suggesting that MecC is directly involved in DCM transformation.

Transformation of dichloromethane by recombinant Mec proteins

To determine if MecC serves as the active methyltransferase responsible for the first step of DCM transformation, we heterologously expressed MecC in *E. coli* BL21 (DE3) and conducted methyl viologen-based *in vitro* enzyme activity assays with the recombinant MecC. With MecC as the only catalyst, no DCM transformation was observed (Figure 2A). Following this, we also heterologously produced MecB and MecE, which were suggested to catalyze DCM transformation (23). These authors proposed that MecE catalyzes the initial cleavage of DCM and transfers the methyl group to the cobalamin bound to MecB. However, also with heterologously produced MecE we did not observe DCM transformation (Figure 2A). Combinations of MecC with MecB (Figure 2A), or MecE with MecC (data not shown), did also not show DCM transformation. In contrast, when MecE and MecB were combined, there was a conversion of DCM at equimolar amounts to the MecB used in the setup, resulting in the transformation of 5 µM of DCM from the initial 50 µM in the reaction.

Complete transformation of DCM was achieved when all three enzymes (MecB, MecC and MecE) were present in the activity master mix (Figure 2A; lower right graph, $MecEBC_{Ac}$). The transformation of DCM with MecB, MecC and MecE was much higher than equimolar with MecB amounts as described above for the activity assays with MecE and MecB. Therefore, we investigated

the fate of the methyl group and searched for a methyl group acceptor in the reaction. The assay solution contained acetate in the buffer, and accordingly, we detected large amounts of acetate by mass spectrometry in all analyzed samples (Figure 3B, peak at *m/z* 59.0134). However, we detected that acetate underwent methylation to form methyl acetate (MeOAc) solely in samples containing MecEBC and acetate ($MecEBC_{Ac}$), as evidenced by a new signal at $[M+H]^+$ = 75.0443 *m/z* (Figure 2C). Given that the activity assay was conducted in a 100 mM acetate buffer containing only 50 µM DCM, the mass signal for acetate was notably higher than that for methyl acetate. In the no-enzyme control (NEC), acetate remained unmethylated, showing only the MS signal for acetic acid at $[M-H]^-$ = 59.0134 *m/z*. Acetic acid (Ac) was detected in the negative ionization mode, while MeOAc was measured in the positive ionization mode (Figure 2B–C).

The results unambiguously demonstrated that acetate is used by the methyltransferase system of *D. formicoaceticum* as a methyl group acceptor. Considering the homology between MecC from *D. formicoaceticum* with MtaA from methanogenic archaea (27), which utilize coenzyme M (CoM) as a methyl group acceptor *in vivo*, we also tested CoM as an artificial methyl group acceptor. In the presence of CoM and recombinant MecE, MecB and MecC, complete transformation of DCM occurred after 18 h of incubation (Figure 2A), accompanied by the formation of methyl-CoM (MeCoM), as indicated by the mass signal at $[M+H]^+$ = 156.9908 *m/z* (Figure 2E). CoM exhibiting a signal at $[M-H]^-$ = 140.9684 m/z was not completely converted to methyl-CoM because of the higher CoM concentration (100 µM) compared to the DCM concentration (50 µM DCM) in the assay (Figure 2E). In the NEC_{CoM} containing 100 µM CoM and 5 µM cyanocobalamin, a minor amount of CoM was abiotically converted to methyl-CoM, indicated by the $[M+H]^+$ = 156.9907 *m/z* signal in the mass spectrum (Figure 2D), even though no significant change was observed in the GC-MS signal for DCM. Moreover, the mass signal for methyl-CoM at $[M+H]^+$ = 156.9907 *m/z* was absent in the CoM stock solution used for the activity assays (Supplementary Figure 6). Given the low cyanocobalamin concentration in the NEC_{CoM} and the abiotic reaction of cyanocobalamin with DCM

observed *via* spectrophotometric and mass spectrometric measurements, which is described in the next section (Figure 3), abiotic demethylation of DCM by cyanocobalamin and CoM is plausible here.

Abiotic reaction of dichloromethane with cyanocobalamin

Spectrophotometry and liquid chromatography coupled to mass spectrometry (LC-MS) were applied to investigate whether the methyl group of DCM interacts abiotically with the corrinoid cofactor under reducing conditions. Non-reduced cyanocob(III)alamin, with or without DCM, had a maximum absorbance peak at 360 nm (Figure 3A). The observation that the peak was unchanged in the presence of DCM indicates that DCM did not interact with the corrinoid in its oxidized Co(III) state (Figure 3A, red dashed line). Reduced cyanocobalamin in its Co(I) state typically displays an absorption maximum at around 390 nm under aqueous conditions, which was observed when reduced cob(I)alamin was incubated without DCM (Figure 3A, green bold line). However, upon incubation of DCM with cob(I)alamin, the absorption maximum at 390 nm (indicative of the Co(I) state) decreased, while the absorption at 520–550 nm increased (indicative of the Co(III) state). This indicates that Co(I) was oxidized to Co(III) due to the interaction between DCM and cob(I)alamin (Figure 3A, orange dashed line) under abiotic conditions. Moreover, LC-MS analysis detected both, methyl cobalamin (MeCbl, $[M+2H]^{2+}$ = 672.8055 *m/z* and $[M+H]^{+}$ = 1344.6003 *m/z*), and chloromethyl-cobalamin (MeClCbl, $[M+2H]^{2+}$ = 689.7819 *m/z* and $[M+H]^{+}$ = 1378.5598 *m/z*) in assays containing reduced cob(I)alamin and DCM. In contrast, in assays without DCM, only cyanocobalamin (Cbl, $[M+2H]^{2+}$ = 678.2959 *m/z* and $[M+H]^{+}$ = 1355.5723 *m/z*) was identified (Figure 3B). These results show that direct interaction between DCM and the reduced corrinoid in the Co(I) state is feasible, leading to the removal of one or two chlorine atoms (Figure 3B), indicating that cob(I)alamin, which is a strong nucleophile, can abiotically demethylate DCM. However, to subsequently transfer the methyl group to a methyl group acceptor a strong nucleophile such as a thiol group in CoM (Figure 2C, upper graph) is necessary.

Purification, characterization and analysis of protein-protein-interactions of recombinant Mec proteins

The properties of MecB, MecC, and MecE were investigated with proteins that were heterologously expressed in *E. coli* BL21 (DE3) and purified using immobilized metal affinity chromatography (IMAC) *via* a polyhistidin tag (His-tag). The monomeric form of MecB exhibited an estimated molecular mass (MM) of approximately 24 kDa, while MecC and MecE exhibited MMs of around 45 kDa and 40 kDa, respectively, as determined by SDS-PAGE (Supplementary Figure 7A). MecB, MecC, and MecE were identified through nLC-MS/MS analysis of the respective gel bands excised from SDS-PAGE. MecC and MecE were identified with a coverage of about 90% and over 870 peptide-spectrum matches (PSMs), while MecB was detected with a 65% coverage and over 550 PSMs. We also conducted pulldown assays using His-tagged MecC or MecE proteins in an attempt to co-purify untagged MecB. First, soluble protein fractions containing tagged recombinant MecC or MecE, obtained from *E. coli*, were bound to the IMAC column. Subsequently, soluble protein extracts containing recombinant and untagged MecB were loaded onto the same column. The standard IMAC purification procedure was then followed, which included two washing steps and elution of the proteins with 250 mM imidazole buffer. The elution fractions of both pulldown purifications were analyzed using SDS-PAGE. However, MecB could not be co-purified with MecC (Supplementary Figure 7B), while it co-eluted with MecE as evidenced by the protein band at approximately 24 kDa (Supplementary Figure 7C). The three suspected gel bands were cut and the identity of MecB, MecC and MecE were confirmed through protein mass spectrometry. During the pulldown purification, MecE was identified with an approximate coverage of 86% and over 820 PSMs, while MecB was detected with a 66% coverage and over 100 PSMs (Supplementary Figure 7C).

To evaluate metal cofactor binding to MecC and MecE proteins, we quantified seven metal ions by ICP-QqQ-MS (Mg, Ca, Fe, Co, Ni, Cu, Zn). Zinc was detected in significant quantities bound to

MecC and MecE, with a value of 3.2 ± 0.1 zinc ions per MecC protein and 1.3 ± 0.2 zinc ions per MecE protein. Other metals (Mg, Ca, Fe, Co, Ni, Cu) were detected at very low quantities, not exceeding 0.2 ions per protein.

To evaluate if the recombinant Mec proteins after IMAC purification were present as monomers, homooligomers or heterooligomers, we separated native protein complexes by BNE (Figure 4A) and SEC (Supplementary Figure 8A). Post-IMAC purification, MecB was observed in both monomeric and dimeric forms *via* BNE (Figure 4A, gel bands 1 and 2), although SEC predominantly showed a monomeric form (Supplementary Figure 8A, peak 3-2). Conversely, MecC appeared as a multimer consisting of three to eight subunits, as indicated by SEC (Supplementary Figure 8A, peak 1) or BNE analysis (Figure 4A, gel band 3), respectively. This might not correspond to the native *in vivo* state of MecC, but rather shows an oligomerization effect due to the very high concentration after heterologous production in *E.* coli and protein concentration step using Amicon filters. However, MecE was predominantly detected as a monomer or homodimer *via* BNE (Figure 4A, gel bands 4 and 5), contrasting with SEC results, where MecE were primarily observed as homotrimers (Supplementary Figure 8A, peak 2-2).To investigate potential protein-protein interactions among MecB, MecC, and MecE and the conditions influencing these interactions, we employed BNE (Figure 4) and SEC (Supplementary Figure 8B). Protein mixtures comprising MecB, MecC, and MecE in a 1:1:1 ratio were mixed and separated under anoxic conditions using BNE (Figure 4A) as well as under oxidizing and reducing conditions (incubating with 0.5 mM titanium(III)citrate for 30 min) *via* SEC (Supplementary Figure 8B). However, when MecB, MecC, and MecE were incubated in a 1:1:1 molar ratio under anoxic and non-reducing conditions, two faint new protein bands at approximately 150 kDa and 60 kDa appeared in the BNE gel (Figure 4A, gel bands 6 and 8). Gel band 6 (~150 kDa) exhibited MecE and MecB in an approximate 1:1 ratio, as determined *via* nLC-MS/MS, suggesting the potential formation of a heterotetrameric $Mec(BE)_2$ complex with an estimated molecular mass (MM) of ~128 kDa. Additionally, a small fraction of MecC was identified

in gel band 6, indicating the possibility of Mec$(BE)_2C$ complex formation with a MM of ~173 kDa. However, the formation of a minor fraction of a homotetrameric $MecE_4$ complex (~160 kDa) or disaggregation of the polymeric MecC structure instead of Mec$(BE)_2C$ cannot be ruled out. In gel band 8, with an approximate MM of 60 kDa, MecB and MecE were detected in an approximate 3:1 ratio, indicating the potential formation of a heterodimeric MecBE complex with a MM of approximately 64 kDa. Similar observations were made for the gel band 9, located directly beneath band 8. However, a complete shift after combining MecB, MecC, and MecE was not observed, indicating either highly dynamic interactions among the three proteins dependent on factors such as the redox conditions of cofactors and substrate binding, or weak interactions between the subunits. Moreover, the presence of the His-tag could also significantly influence interactions. Evidence that protein-protein interaction may be influenced by the redox state of cofactors (particularly by cobalamin in MecB) is apparent when mixtures of MecB, MecC, and MecE were incubated and separated under reducing conditions (Supplementary Figure 8B), resulting in a distinct shift of elution peaks to two main peaks.

Structure prediction of MecB, MecC, and MecE using AlphaFold2

To obtain information on the three-dimensional structure, mechanism and functionality of the MecB, MecC, and MecE proteins in DCM transformation within *D. formicoaceticum* strain EZ94, various computational analyses were undertaken.

MecB (213 amino acids, 23 kDa) shares complete amino acid identity with the MecB protein encoded in *D. formicoaceticum* strain DMC. According to the AlphaFold2 predicted structure, MecB has two domains: The N-terminal extension comprises four α-helices and is connected *via* a 12-amino acid linker to a Rossmanoid domain, comprised of five central β-sheets surrounded by five α-helices at the C-terminal end. Within the Rossmanoid domain of MecB, a highly conserved cobalamin binding motif **D**X**H**X_2**G** was identified, which is characteristic for proteins with corrinoid bound in a dimethylbenzimidazole (DMB) base-off/His-on configuration (28,29). The conserved histidine

residue His103 of MecB interacts with the cobalt atom of the cobalamin cofactor in the calculated structure (Supplementary Figure 9). During the catalysis, the cobalt atom in the cobalamin cofactor might undergo cycling through various conformations: a penta- or hexa-coordinated base-off/His-on conformation in catalytically inactive cob(II)alamin or methyl-cob(III)alamin, and a base-off/His-off in cob(I)alamin, where the cobalt atom is tetra-coordinated. However, a tight binding of cob(I)alamin, even in the base-off/His-off state, is confirmed for other corrinoid-dependent proteins, facilitated by the strong binding of the DMB tail by highly conserved hydrophobic amino acid residues as has been already shown for other cobalamin-dependent proteins, *e.g.*, MetH and MtaC (30). In the case of MecB, these residues include Ile105, Gly106, Ile109, Val110, Gly179, and Gly180 (Supplementary Figure 9).

Despite only a 35% amino acid sequence identity, the *in silico* tertiary structure of MecB, calculated with AlphaFold2 with an overall confidence score of 92.3%, resembles the MtaC crystal structure (Supplementary Figure 9, grey cartoon). MtaC, a cobalamin-dependent methyltransferase from *Methanosarcina barkeri*, is responsible for transferring the methyl group from methanol to coenzyme M (CoM) with the aid of two methyltransferases (MTs). While the demethylation of methanol is catalyzed by the MT MtaB, the methylation of CoM to methyl-CoM is performed by a second MT MtaA. Both MecB and MtaC share similar architectural features. They bind a corrinoid cofactor as a prosthetic group, exposing the upper ligand site of the cofactor for methylation by MT I and demethylation by MT II. Additionally, both MecB and MtaC contain an N-terminal extension, with highly conserved amino acid residues (in MecB: Gly56, Phe59, Leu60, Pro61), which may play a role in the interaction and complex formation with an MT I as reported by previous studies (31,32).

For MecC (386 amino acids, 44 kDa) and MecE (337 amino acids, 38 kDa), the CDART server analysis suggested the presence of a uroporphyrinogen decarboxylase (URO-D) domain. Analysis of the *in silico* tertiary structures of MecC (confidence score of 89.6%) and MecE (confidence score of 98.7%), calculated using AlphaFold2, revealed a TIM-barrel-like conformation (Figure 5). Both

MecC and MecE are cobalamin-independent enzymes and are structurally very similar, with an RMSD of approximately 1.6 Å when superimposed. Furthermore, their tertiary structures closely resemble that of MtaA from *M. mazei* (27) (PDB: 4AY7) with RMSD values of approximately 2.2 Å or 1.6 Å, respectively. However, despite this structural similarity, MecC shares only about 24% amino acid sequence identity with MtaA, while MecE shares approximately 26%. Notably, MecE is structurally and sequentially divergent from MtaB (PDB: 2I2X), the enzyme responsible for the demethylation of methanol in the methanol:cobalamin methyltransferase complex of *M. barkeri* (33), with RMSD of approximately 21.4 Å and no significant sequence similarity. Unlike MtaA, the crystal structure of which reveals a zinc ion coordinated by the conserved zinc binding motive **H**X**C**X_n**C** in the center of the funnel, such a motif was not found in MecC. Instead, ICP-MS data suggest that three zinc atoms are bound to the structure. Using multiple sequence alignment of ten homologous MecC proteins and the ZincBind webserver three consensus motifs with conserved amino acid were identified in the periphery of the MecC model, potentially responsible for zinc binding: (*i*) Glu238, His194 and probably Glu198 (not strictly conserved); (*ii*) Asp39, Cys345, Cys349, and probably His37 (not strictly conserved), and (*iii*) Cys59, Asp65 and His69 (Figure 5B). Calculated distances between the possible coordinating amino acids and putative zinc atoms were mostly <3.0 Å (Figure 5C), indicating significant zinc-binding potential (34). In contrast, the highly conserved zinc binding motif **H**X**C**X_n**C** was identified in the primary structure of MecE *via* multiple sequence alignment, with residues Cys 193, His225, Cys227, and Cys312. These likely coordinate the zinc atom with distances less than 2 Å (Figure 5D). Additionally, metal quantification in recombinant MecE using ICP-MS indicates that only one zinc is bounded to MecE.

Subsequently, we calculated the structure of a heterodimer MecBC (Supplementary Figure 10) and MecBE (Figure 6) using AlphaFold2 with overall confidence score of 82.0% and 84.0%, respectively. In the case of the calculated MecBC heterodimer, cobalamin of MecB is directed towards the zinc-free funnel of MecC, while the predicted zinc ions are bound in the periphery, at

distances disabling direct interaction with MecB's cobalamin. To enable interactions between the cobalamin and zinc atoms of MecC, large protein movements are necessary (Supplementary Figure 10). The *in silico* predicted structure of MecBE forms a heterodimer, where the cobalamin at the top of the Rossmanoid fold of MecB is orientated with its open coordination site facing the zinc ion of MecE. This zinc ion is embedded in a funnel-shaped pocket of MecE (Figure 6). The calculated distance between the zinc ion in MecE and the cobalt atom of cobalamin in MecB amounted to approximately 8.2 Å (Figure 6).

DISCUSSION

Our experiments, combining BNE, *in vitro* enzyme assays utilizing both wild type and recombinant enzymes from strain EZ94, along with mass spectrometry and computational analyses, strongly support previous observations (23) that Mec proteins encoded by the *mec* gene cassette are pivotal for DCM transformation. In our work, MecC was the most abundant protein in the crude extract, followed by MecB, MecE, and MecF. In the subsequent sections, we will discuss the specific roles of each protein and their involvement in the first steps of DCM transformation.

The cobalamin-dependent step plays a pivotal role in DCM transformation

In the fermentation of DCM by *D. formicoaceticum* strain DMC, a cobalamin-dependent protein has been proposed to play a pivotal role in DCM transformation, supported by the light-reversible inhibition of the reduced cobalamin protein with propyl iodide (15). Our biotic investigations using crude protein extracts align with these prior findings for *D. formicoaceticum*, as evidenced by the inhibition of DCM transformation by propyl iodide (Supplementary Figure 2) and the characteristic cob(III)alamin absorbance detected in the active protein fractions separated by anion exchange chromatography (Supplementary Results, Supplementary Figure 3). Furthermore, our abiotic experiments reveal that under strongly reducing conditions, reduced cob(I)alamin can undergo

nucleophilic attack of the methyl group in DCM, thereby catalyzing the transformation of DCM into chloromethyl-cob(III)alamin and methyl cob(III)alamin (Figure 3). Among the highly expressed cobalamin-dependent enzymes from the *mec* operon, MecB emerges as the most plausible candidate involved in DCM transformation in *D. formicoaceticum*. BNE gel activity assays experiments showed significant DCM transformation in slices where MecB was present despite not being the most abundant protein (Supplementary Figure 5). Moreover, in activity assays with recombinant Mec proteins, DCM transformation occurred in stoichiometric amounts with the amount of MecB present in the reaction when only MecB and MecE were tested together (further details are provided in the subsequent section). Nevertheless, these findings indicate that MecB likely serves as a shuttle, facilitating the transfer of the methyl group between an enzyme demethylating a methyl group donor or methylating a methyl group acceptor. It may also function as a reservoir of binding and temporary holding the methyl group until it is used by a methyltransferase. A better annotation for this protein might therefore be a "corrinoid-dependent methyl shuttle protein".

MecC and MecE methyltransferases are responsible for DCM transformation *in vitro*

Recent studies have identified Mec proteins encoded by the *mec* operon as potential catalysts for DCM transformation across various strains of *D. formicoaceticum*, including strains EZ94 and DMC, as well as '*Ca.* Dichloromethanomonas elyunquensis', *'Ca.* Formimonas warabiya', and *Dehalobacter* (20, 21, 23, 25). Comparative genomics suggests that the *mec* gene cassette lacks close homologs in publicly available bacterial genomes, except for methanogenic archaea utilizing C1-compounds such as methanol as a carbon source (27,33). Sequence analysis reveals that MecB of strain EZ94 shares approximately 35% amino acid similarity with MtaC of *M. barkeri* and resembles it structurally, while MecE and MecC closely resemble MtaA from *M. mazei*. In methanogenic archaea, a methyltransferase shuttle system comprising MtaB, MtaC, and MtaA facilitates the production of methyl-CoM from various methyl donors (33). However, while the structures of MecB, MecC, and MecE bear resemblance to enzymes in this system, there is no evidence of methyl-CoM

and methane formation from DCM fermentation (14,15,18,19,24). Murdoch et al. (23) suggested that the methyl transfer system responsible for DCM transformation in *D. formicoaceticum* involves MecE and MecF as methyltransferases I and II (MT I and MT II), respectively, with MecB acting as corrinoid protein (CoP). Another study aligns with Murdoch's proposal that MecE is responsible for DCM demethylation, although other methyltransferases such as MecC and MecI were identified as putative MT I enzymes (21). For these two proteins, no function was attributed. However, our BNE experiments show that MecC is the most abundant protein in slices with the highest DCM transformation activity, co-eluting with MecF, while MecB and MecE were less abundant (Figure 1, Supplementary Figures 4 and 5). Moreover, MecB and MecE co-elute at high relative abundance in slices where no DCM transformation is detected, questioning their previously hypothesized role in DCM dechlorination (Figure 1). Thus, our data indicate that particularly MecC plays the pivotal role in DCM transformation, despite not being included in the methyl transfer system proposed by others (23). However, the analysis of MecC's *in silico* tertiary structure does not fully support this hypothesis, but reveals important characteristics of this protein related to DCM transformation. MecC appears to bind three zinc ions within its structure, all situated in the periphery rather than within the funnel. This configuration makes it less likely to form a stable complex with MecB, which might be crucial for DCM demethylation (Supplementary Figure 10).

In contrast, the *in silico* structure of MecE shows one zinc ion located in the center of the TIM-barrel structure. This positioning could allow direct interaction with the corrinoid cofactor of MecB at approximately 8.2 Å of distance, favoring the binding of a small substrate, such as DCM (Figure 2 and 6). Furthermore, the results of protein-protein interaction studies using both recombinant and wild type enzymes, conducted through SEC and BNE, indicate that MecB forms a stable complex primarily with MecE rather than MecC (Figures 1 and 4, Supplementary Figure 4 and 8). Additionally, during IMAC purification, untagged MecB co-elutes with recombinant MecE, which carried a His-tag. This co-elution phenomenon was not observed for MecC (Supplementary Figure

7). Therefore, our collective findings from BNE, SEC, and IMAC purification attempts strongly suggest that MecB might rather form a stable complex with MecE necessary for demethylation of DCM and thus, MecE acts as MT I, while MecC likely serves as MT II, catalyzing the transfer of the methyl group onto an acceptor molecule.

In line with BNE results, activity assays employing recombinant Mec proteins expressed in *E. coli* revealed that complete *in vitro* transformation of DCM occurred only when MecB, MecC and MecE were combined. Notably, when MecB and MecE were mixed, a stoichiometric amount of DCM, relative to the quantity of MecB in the reaction setup, was transformed, suggesting that DCM transformation ceased due to the complete methylation of MecB by MecE (Figure 2A). These results strongly suggest that MecE indeed carries out the demethylation of DCM, transferring the methyl group from DCM to the cobalamin bound to MecB. However, the cessation of demethylating activity by MecE and MecB in the absence of MecC indicates that MecC is involved in the removal of the methyl group from MecB's methyl cobalamin, thereby making the cobalamin available for the next methylation cycle. In the *in vitro* activity assays, MecC was observed to transfer the methyl group either to acetate or to CoM, as confirmed by mass spectrometry. In contrast, when MecC was absent, neither acetate nor CoM was methylated (Figure 2B–C).

Taken together, these findings indicate that *in vitro* DCM transformation using recombinant Mec enzymes depend on the presence of MecC. Moreover, we suggest that even in activity assays conducted with BNE slices, the demethylating activity of MecC was decisive to monitoring the DCM transformation, given that MecE and MecB alone were not sufficient to reveal DCM transformation in the gel slices. Therefore, our outcomes indicate that MT II enzyme (MecC) facilitates the removal of the methyl group from MecB's methyl cobalamin *in vitro*.

Proposed DCM transformation mechanism by MecEBC complex of *D. formicoaceticum*

Our data strongly supports the formation of a stable MecBE heterodimer, which may further dimerize into a $(MecBE)_2$ heterotetrameric complex (Figures 4 and 6, Supplementary Figure 7), consistent with previous research (31). MecBE might then undergo dynamic interactions with MecC (Figure 7 and Supplementary Figure 11), potentially influenced by the oxidation state of MecB's cobalamin or its interactions with other protein partners. This observation aligns with our BNE results, revealing dissociation of MecC from MecBE and resulting in the highest DCM transformation activity exclusively in BNE slices containing high amounts of MecC and low amounts of MecBE. We hypothesize that even minor quantities of MecB and MecE suffice to drive the demethylation of DCM, provided that a sufficient amount of MecC is present. Considering that the *in silico* structure of MecC predicts three zinc ions, it is possible that MecC may possess multiple active sites capable of accepting various methyl group acceptors, which are subsequently methylated by MecB's methyl cobalamin. However, it is also conceivable that two of the three zinc ions in MecC may have stabilizing or structural role rather than a catalyzing function, as has been evidenced for other proteins (35-37). Our current data cannot conclusively differentiate these possibilities.

Overall, our data, including abiotic experiments, suggest that cob(I)alamin within MecB initiates nucleophilic attack on the chloromethyl group of DCM, coordinated by the zinc of MecE (MT I), resulting in chloromethyl-cob(III)alamin bound to MecB and chloride release. Subsequently, MecC (MT II) coordinates a nucleophilic methyl group acceptor at the catalytically active zinc, enhancing its nucleophilicity. This enables it to attack the methyl group of chloromethyl-cob(III)alamin in MecB. During this process, a second chloride is released, and the methyl group is likely protonated by water (Figure 7). The exact nature of the second cleavage *in viv*o, whether it is a homolytic cleavage releasing cob(II)alamin in MecB or a heterolytic cleavage releasing cob(I)alamin, remains unclear. Further investigations are required in this regard. If the cleavage is homolytic, it would

necessitate reactivation of the catalytically inactive cob(II)alamin to cob(I)alamin bound to MecB, potentially facilitated *in vivo* by MecH.

Finally, our experiments demonstrate that acetate and CoM can function as methyl group acceptors for MecC *in vitro*. However, *in vivo*, it is expected that the chloromethyl group bound to MecB is transferred to THF, forming methylene-THF *via* MecF, which is reduced to methyl-THF entering the Wood-Ljungdahl pathway (23). Since MecC does not have a THF binding motif, we hypothesize it cannot methylate THF. However, we did not test THF as methyl group acceptor *in vitro* and cannot exclude this possibility. *In vivo*, MecC might play an important role as a versatile methyltransferase, capable of conducting *O*- and *S*-methylations of various methyl group acceptors, thereby introducing the methyl group into different C1-pathways of *Dehalobacterium.* This function could be important in the biosynthesis of amino acids (*e.g.*, *L*-methionine), cofactors (*e.g.*, *S*-adenosylmethionine), and the methylation of nucleotides. Identifying the *in vivo* methyl group acceptors of MecC was beyond the scope of our study and cannot be determined from our data. Future studies should address this important question.

Additionally, we cannot exclude the interaction of MecBE with other methyltransferases such as MecF, which could potentially transfer the methyl group to other acceptors like tetrahydrofolate. Consequently, further investigations are needed to elucidate the specificity and precise mechanism of MecC, as well as the roles of other Mec proteins within the *mec* cassette, such as MecF.

MATERIALS AND METHODS

Cultivation, cell harvesting, and preparation of protein extracts

For wild type protein experiments, we used a *Dehalobacterium*-containing mixed culture, obtained from slurry samples of the membrane bioreactor of an industrial wastewater treatment plant (24). This culture was grown in 100 mL glass serum bottles, each containing 65 mL of reduced, anoxic, bicarbonate-buffered mineral medium (pH 7), as described elsewhere (25). The active DCM

transforming culture was maintained for more than five years by periodic transfers every 10–15 days. Quantification of chlorinated methanes in the 100 mL serum bottles was carried out by injecting 0.5 mL headspace samples into a gas chromatograph equipped with a flame ionization detector (GC-FID), as described elsewhere (24). To obtain cell concentrates, protein crude extracts and soluble protein fractions, cells were harvested from 210 mL of the culture containing *D. formicoaceticum* strain EZ94 during the exponential transformation phase after consuming approximately 3 mM DCM. This was achieved by centrifugation at 9,000 x *g* for 1 h at 16 °C, removing 90% of the supernatant, and then resuspending and combining the remaining volume. This centrifugation process was repeated, and the final pellet was resuspended in 1 mL of a sterile, anaerobic buffer solution containing 100 mM PBS and 1 mM *L*-cysteine as a reducing agent.

The crude protein extract from the *Dehalobacterium*-containing culture was obtained by lysing the cell concentrate with 100 mg mL^{-1} lysozyme at 37 °C for 30 min. Subsequently, two rounds of bead beating (FastPrep FP120, Thermo) were performed at a speed of 6.5 m s^{-1} for 35 s, with intervals of 1-min cooling on ice. The lysed cells were then centrifuged at 6,000 x *g* to remove cell debris. Further separation of proteins into membrane and soluble protein fractions was achieved by ultracentrifugation (Optima MAX-XP Ultracentrifuge, Beckman Coulter) at 120,000 x *g* for 1 h at 4 °C. The membrane protein fraction (pellet) obtained was resuspended in a buffer solution containing 100 mM PBS and 1 mM *L*-cysteine. The soluble fraction of the protein extract (supernatant) from the enriched culture underwent size-based separation and concentration using Amicon ultrafilters with exclusion sizes of 100 kDa and then 30 kDa (Amicon Ultra, 0.5 mL Centrifugal Filters, Millipore). This involved centrifugation at 16 °C for 5 min at 14,000 x *g*, with recovery of concentrated soluble proteins by inverting the filters and centrifuging for 2 min at 1,000 x *g* and 16 °C. Three soluble protein fractions with the following molecular mass sizes were obtained: one above 100 kDa, another between 30 and 100 kDa, and a third below 30 kDa. The protein content was determined by measuring the absorbance at 280 nm using a nanodrop.

560 **Abiotic dechlorination of dichloromethane with reduced cyanocobalamin**

561 The initial abiotic test was conducted in a microtiter plate with spectrophotometric measurements of
562 the UV-Vis spectrum in the range between 300 and 700 nm (0.5 nm steps) during the reaction inside
563 an anaerobic chamber (Coy Laboratories, USA). Cyanocobalamin, titanium(III)citrate, and DCM
564 were used in the reaction at a concentration of 0.5 mM each, and the total reaction volume was
565 200 µL. Four conditions were examined: *i)* cyanocobalamin reduced with titanium(III)citrate for 1 h
566 without DCM, *ii)* cyanocobalamin reduced with titanium(III)citrate for 1 h with DCM addition, *iii)*
567 non-reduced cyanocobalamin with DCM, and *iv)* non-reduced cyanocobalamin without DCM. The
568 reaction started with the addition of DCM, when indicated, and the UV/Vis spectrum was recorded
569 for 10 min. An iteration of the abiotic tests for conditions *i)* and *ii)* was performed using increased
570 concentrations of cyanocobalamin, titanium(III)citrate, and DCM of 0.75 mM each in 2 mL vials,
571 resulting in a total reaction volume of 400 µL. The compounds formed in these reactions were
572 analyzed with a high-performance liquid chromatography (HPLC) system (Dionex 3000 Ultimate,
573 Thermo) coupled to tandem mass spectrometry Orbitrap Fusion (MS/MS, Thermo). Therefore, liquid
574 samples were diluted with ddH_2O to a total volume of 2 mL and were filtered through membrane
575 filter with a pore size of 0.22 µm (Millex-GV, PVDF, Millipore). A Dionex autosampler injected 25
576 µL of the sample into the HPLC system equipped with a LiChrospher 100 RP-18 (5 µm)
577 LiChroCART 125-4 column. The column temperature was maintained at 25 °C, and the separation
578 was carried out isocratically for 60 min at a flow rate of 0.2 mL min^{-1} using methanol and 0.1% (v/v)
579 formic acid as the mobile phase.

580 **Protein separation by blue native gel electrophoresis**

581 Blue native gel electrophoresis (BNE) was conducted inside the anaerobic chamber using soluble
582 protein fractions with a size above 100 kDa from the *Dehalobacterium*-containing culture. A volume
583 of 30 µL containing 20 µg or 40 µg protein extracts supplemented with 0.125% (w/v) Coomassie G-
584 250 additive was loaded into each well of a pre-casted 4–16% gradient Bis-Tris gel (NativePAGE

585 Novex, Invitrogen). The electrophoresis was run inside the anaerobic chamber at 150 V for 1 h,
586 followed by an increase in voltage to 250 V for an additional 30 min. The system was maintained at
587 a low temperature using ice packs. The NativeMark Unstained Protein Standard (Invitrogen) was
588 used as the protein ladder. Anode buffer and "light blue cathode buffer" required for the
589 electrophoresis were prepared according to the manufacturer's instructions, degassed, and chilled to
590 4 °C before use. After the electrophoresis run, two replicate lanes loaded with 20 µg and 40 µg of
591 soluble protein fraction together with two protein ladders were separated from the rest of the gel.
592 These four lanes were silver-stained using a mass-spectrometry compatible procedure (38).
593 Additionally, triplicate lanes loaded with a minimum of 2,200 µg of soluble protein extract from the
594 *Dehalobacterium*-containing culture were not stained; they were kept cold and wetted with anode
595 buffer until they were cut into slices using a scalpel that was cleaned with anoxic sterile water after
596 each cut. The cuts were guided by the protein ladder and the protein bands observed in the parallel
597 silver-stained lanes. The unstained gel slices were placed in individual 10 mL GC crimp vials for
598 enzymatic assays. The two stained gel lanes were used to identify proteins through protein mass
599 spectrometry and were cut into slices of equal size for subsequent in-gel trypsin digestion.

600 **Enzymatic assays with wild type proteins**

601 The enzymatic transformation of DCM was set up inside the anaerobic chamber using 10 mL GC
602 crimp vials. The reaction mixture, with a final volume of 0.5 or 1 mL, included 100 mM potassium
603 acetate buffer (pH 5.8), 0.5 mM methyl viologen as electron donor, 1 mM titanium(III)citrate as
604 reducing agent, and either the protein fraction or the gel slice. Vials were sealed with Teflon-coated
605 rubber septa and aluminum crimps, then amended with 45 µM DCM from acetone stock solutions
606 using a Hamilton glass syringe. Subsequently, the vials were vortexed and incubated upside down in
607 the dark at room temperature. After 24 h, transformation of DCM was analyzed by gas
608 chromatography. For all enzymatic assays, controls were added in triplicates. Abiotic controls
609 comprised 1 mL of the reaction mixture amended with 45 µM of DCM, excluding the addition of

cells, protein extracts, or blue native gel slices. In the case of the activity assays conducted with the gel slices post-blue native gel electrophoresis, three additional controls were included: *i)* a positive control in which the soluble protein fraction replaced the gel slices, *ii)* another positive control containing soluble protein fraction supplemented with 0.125% (w/v) Coomassie G-250 and sample buffer, and *iii)* a negative control that contained pellet of cell debris after cell lysis to evaluate cell disruption performance. All the controls were set up in the reaction mixture and subsequently incubated at room temperature for 24 h.

Bioinformatics and protein structure prediction

The genomic assembly of *D. formicoaceticum* strain EZ94 has been previously published (25), with the sequenced and annotated genome available on the Microscope platform under ID 13191 (https://mage.genoscope.cns.fr/microscope/), and deposited at NCBI under accession number GCA_024705665.

Protein structure prediction for MecB, MecC, and MecE proteins of *D. formicoaceticum* strain EZ94 was conducted using the AlphaFold2 ColabFold platform (39). Structure visualization, refinement, and superposition of protein structures was done with PyMOL 2.5.4 (40). Cofactor binding sites were predicted with the COFACTOR server (41,42), while I-TASSER was used for calculating ligand-binding sites and predicting protein functions based on the obtained structures (43-45). The ZincBind webserver aided in the prediction of zinc binding sites (34). The identification of conserved amino acid residues involved multiple sequence alignments of MecB, MecC, and MecE homologs from various microorganisms, which was carried out using MEGA 11 (46).

Construction of expression vectors

Genomic DNA from the *Dehalobacterium* strain EZ94-containing mixed culture was isolated using the DNeasy PowerSoil Pro Kit (Qiagen), following the manufacturer's instructions. Expression plasmids pET22B:MecE, pET22B:MecB, and pETDuet:MecC, were constructed for this study. To

integrate *mecE* and *mecB* from strain EZ94 into the pET22B vector, the plasmid was linearized with NdeI and BamHI or NdeI and NcoI, respectively. For the cloning of *mecC*, the pETDuet vector was linearized with BamHI and SacI. For the digestion process FastDigest restriction enzymes (Thermo Fisher Scientific) were used, following the manufacturer's instructions. Concurrently, *mecE*, *mecB*, and *mecC* genes were PCR-amplified using the CloneAmp HiFi PCR Premix (Takara Bio) and primers with 15-nucleotide overhangs identical with the insertion sites of the vector (indicated below by the underlined sequence). For *mecE* amplification, the forward primer 5'-aag gag ata tac ata tga att cga gag aga gag ttt ttg c-3' and reverse primer 5'-gct cga att cgg atc ctc gta ccg ccc aaa ttt ttc tg-3' were used; for *mecB*, the primers were 5'-aag gag ata tac ata tga gca aaa aaa ttt tag aac-3' and 5'-att ccg ata tcc atg ttc acc ctc cag caa tct tc-3'; and for *mecC*, the primers were 5'-acc aca gcc agg atc tgt cca gta aag agc aaa cgg aa-3' and 5'-cct gca ggc gcg ccg tta tcc caa ttt gct caa att aat g-3'. PCR amplicons and linearized plasmids were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific) directly from solution or from 0.7% (w/v) agarose gels, respectively. Subsequently, PCR fragments were cloned into linearized plasmids using the In-Fusion HD Cloning Kit (Takara Bio), following the manufacturer's protocol. The resulting plasmids pET22:MecE and pET22B:MecB featured a C-terminal polyhistidine-tag fused to the coding sequence. In the pETDuet:MecC vector, MecC carried an N-terminal polyhistidine-tag.

Heterologous production and purification of recombinant proteins

MecE and MecC proteins were heterologously expressed in *E. coli* BL21 (DE3) without the assistance of any auxiliary plasmid. In contrast, MecB production was done in *E. coli* BL21 (DE3) carrying the auxiliary plasmid pBAD:BtuB–F, responsible for cobalamin uptake from the medium (47). Precultures of *E. coli* BL21 (DE3) strains containing either pET22B:MecE or pETDuet:MecC, and *E. coli* BL21 (DE3) carrying pET22B:MecB along with the auxiliary plasmid pBAD:BtuB–F, were set up in Luria-Bertani (LB) medium supplemented with 100 $\mu g\,mL^{-1}$ ampicillin or 100 $\mu g\,mL^{-1}$ ampicillin and 50 $\mu g\ mL^{-1}$ spectinomycin, respectively. These cultures were grown

overnight on a rotary shaker at 37 °C and 140 rpm. On the following day, 1% (v/v) of the overnight cultures was used to inoculate fresh LB medium containing the appropriate antibiotics. The cultures were incubated at 37 °C with agitation at 140 rpm until the OD_{600} reached 0.6–0.8. For strains lacking pBAD42:BtuB–F, induction of MecE and MecC protein expression was initiated with 100 µM IPTG, and the growth medium was supplemented with 1 mM zinc sulfate. In contrast, for the strain producing MecB and carrying pBAD42:BtuB–F, a pre-induction was done with 0.05% (w/v) *L*-arabinose. Subsequently, the culture was supplemented with 5 µM cyanocobalamin and agitated for 1 h at 37 °C. Following pre-induction, expression of *mecB* was induced with 100 µM IPTG. To facilitate slow protein synthesis, thus ensuring correct protein folding and minimizing inclusion body formation, all target proteins were produced for approximately 18 h at 20 °C. The cells were then harvested by centrifugation, and cell pellets were washed with 50 mM Tris/HCl (pH 7.5).

For subsequent protein purification, cell pellets were resuspended in 50 mM Tris/HCl, 10% (v/v) glycerol (pH 7.5), and cells were disrupted at 6 m s^{-1} for 2 x 30 s using 200 µm silica beads by a FastPrep-24TM 5G (MP Biomedicals). Soluble protein fraction was obtained by centrifuging crude extracts at 4 °C and 100,000 x *g* for 1 h (Beckman Optima MAX-XP). Target proteins were purified from the supernatant, containing the soluble protein fraction, using an ÄKTA purifier FPLC system (GE Healthcare Life Sciences) equipped with a 1 mL His60 Ni Superflow column (Takara Bio) through immobilized metal affinity chromatography (IMAC). All purification steps were conducted at a flow rate of 1 mL min^{-1}. The soluble protein fraction was applied to the column, which had been equilibrated with 50 mM Tris/HCl, 10% glycerol (pH 7.5) buffer. After protein binding, the column was washed with five column volumes of the corresponding buffer, followed by a second washing step with 50 mM Tris/HCl, 10% glycerol, 10 mM imidazole (pH 7.5) buffer. The target proteins were eluted with 250 mM imidazole. Protein elution was monitored by measuring absorbance at 280 nm (and 360 nm for MecB). Fractions containing the target proteins, identified by high absorbance at 280 nm and SDS-PAGE analysis, were pooled, and imidazole was removed using a Sephadex G-25

684 containing HiTrap desalting column (Cytiva), replacing the elution buffer with 50 mM Tris/HCl,
685 10% (v/v) glycerol (pH 7.5). Desalted fractions containing the target proteins were pooled and
686 concentrated with Amicon Ultra-4 centrifugal filter units with a molecular mass cutoff of 30 kDa for
687 MecE and MecC and 10 kDa for MecB. Protein concentrations were determined using the BCA assay
688 (Thermo Fisher Scientific) following the manufacturer's protocol.

689 **Sodium dodecyl sulfate polyacrylamide gel electrophoresis**

690 The purity of MecB, MecC, and MecE subunits following purification was assessed through 10%
691 SDS-PAGE analysis. Post-electrophoresis, the gel was stained with a 1 x Coomassie Brilliant Blue
692 R-250 solution comprising 10% (v/v) ethanol, 2% (v/v) acetic acid, and 0.02% (w/v) Coomassie R-
693 250. The gel was initially washed with ddH_2O, then covered with the staining solution and heated in
694 a microwave, followed by incubation for 20 min with coverage. Excessive staining was removed
695 through multiple washing steps with hot ddH_2O, utilizing microwave-assisted heating. For
696 qualitative identification, protein bands corresponding to the respective proteins' molecular masses
697 were excised from the 10% SDS-PAGE gels and prepared for mass spectrometric analysis.

698 **Size exclusion chromatography with recombinant proteins**

699 The interaction between MecB, MecC, and MecE, as well as the oligomeric states of these proteins
700 after heterologous production and IMAC purification, were examined using size exclusion
701 chromatography (SEC) with an analytical Superose 6 Increase 3.2/300 column (Cytiva). To ensure
702 equilibration, the SEC column was equilibrated with a minimum of 1.5 column volumes (one column
703 volume was approximately 2.4 mL) of 50 mM Tris/HCl, 10% (v/v) glycerol (pH 7.5) buffer at a flow
704 rate of 0.03 mL min^{-1}. To assess the oligomeric state of the proteins after IMAC purification, 20 µL
705 samples, each containing 10 µM protein, were injected onto the SEC column and separated at a flow
706 rate of 0.03 mL min^{-1}. Protein interactions were investigated under both oxidizing and reducing
707 conditions. For both conditions, a mixture comprising all three proteins at a 1:1:1 ratio (10 µM each)

was prepared, resulting in a total volume of 20 µL. To ensure reducing conditions, the protein mixture was additionally treated with 0.5 mM titanium(III)citrate before SEC. The mixtures were separated at a flow rate of 0.03 mL min^{-1}. In the case of protein interaction and complex formation, a shift in protein elution peaks was anticipated. The molecular mass of each elution peak was determined relative to the elution volume of the SEC protein standard mix ranging from 15 to 600 kDa (Merck).

Enzymatic assays with recombinant proteins

To examine demethylation of DCM catalyzed by Mec proteins, we conducted enzyme activity assays based on methyl viologen under dim light and strictly anoxic conditions. The assays, with a total volume of 0.5 mL, were set up in 10 mL GC crimp vials. The assay buffer comprised final concentrations of 100 mM potassium acetate buffer (pH 5.8), 1 mM methyl viologen (as an artificial electron donor, reduced with 1 mM titanium(III)citrate) and 5 µM cyanocobalamin. Recombinant proteins MecB, MecC, and MecE were used in different combinations at a concentration of 5 µM each. Coenzyme M at a concentration of 100 µM was used as an artificial methyl group acceptor. After mixing all components, the GC vials were crimped, and the reaction was started by adding 50 µM DCM through a septum using a Hamilton syringe. Following an incubation period of 18 h at 30 °C, the reactions were analyzed by GC-FID (Agilent 7820A, Agilent Technologies). To account for abiotic reactions, no-substrate-controls (NSC) and no-enzyme-controls (NEC) were prepared. NSC contained buffer instead of DCM in the assay buffer, while NEC included buffer instead of enzymes in the suspension. Additionally, we investigated the identities of methylated products in the assay buffer resulting from the demethylation of DCM through mass spectrometry in direct injection mode.

729 **Dichloromethane quantification by gas chromatography-flame ionization**

730 Details regarding DCM quantification in enzymatic assays containing wild type enzymes or
731 recombinant MecB, MecC and MecE proteins are described in the Supplementary Material and
732 methods.

733 **Protein identification by nano-liquid chromatography-tandem mass spectrometry**

734 For in-solution proteomic analysis of different protein fractions from the *Dehalobacterium*-
735 containing culture, the samples underwent reduction of disulfide bridges using 62.5 mM
736 dithiothreitol, followed by alkylation of cysteine residues with 128 mM iodoacetamide.
737 Subsequently, 0.6 µg trypsin (Promega) was used for digestion at 37 °C overnight, with termination
738 of the reaction with 0.1% (v/v) formic acid. The resulting peptides were desalted using C_{18} Zip Tips
739 (Merck Millipore) for subsequent nano-liquid chromatography-tandem mass spectrometry (nLC-
740 MS/MS), as described elsewhere (48).

741 For in-gel trypsin digestion of silver-stained gels and protein mass spectrometry, duplicate silver
742 stained slices from BNE gels were processed. These slices, concurrently used for activity assays,
743 underwent a series of preparatory steps. Initially, they were washed with LC-MS grade ddH_2O and
744 de-stained in a 1:1 (v/v) solution containing 30 mM $K_3[Fe(CN)_6]$ and 100 mM $Na_2O_3S_2$. Protein
745 reduction within the gel slices was achieved with 10 mM dithiothreitol, followed by carbamidylation
746 of cysteine residues with 100 mM iodoacetamide. In the case of in-gel trypsin digestion of
747 Coomassie-stained gel bands, acetonitrile, 10 mM dithiothreitol and 100 mM iodoacetamide were
748 used for destaining, reduction and alkylation of proteins within the protein bands. Subsequently, the
749 proteins were digested with 0.1 µg trypsin (Promega) at 37 °C overnight. The resulting peptides were
750 extracted from the gel pieces and dried in a vacuum centrifuge as previously described (49). Then,
751 the peptides were dissolved in 10 µL of 0.1% (v/v) formic acid, further desalted using C_{18} ZipTip

Pipette Tips (Merck Millipore) and dried in a vacuum centrifuge. Prior analysis, the peptides were resuspended in 20 µL of 0.1% (v/v) formic acid.

Peptide samples obtained from both in-solution and in-gel digestions were analyzed using a nLC-MS/MS composed of a nanoUPLC system (UltiMate 3000 RSLCnano System, Thermo Fisher Scientific) equipped with an Acclaim PepMap 100 C_{18} column (75 µm × 25 cm), coupled to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) *via* an electrospray ion source (TriVersa NanoMate, Advion), following established procedure (48). Proteome Discoverer (v2.4, Thermo Fisher Scientific) was used for the identification of proteins, based on a protein database constructed from the genome sequence of *D. formicoaceticum* strain EZ94 (NCBI accession number GCA_024705665), with SequestHT used as the search engine. Stringent criteria were set with a false discovery rate threshold of 1% for peptide identification using the Target Decoy PSM Validator node. The Minora node in Proteome Discoverer was used for protein quantification through label-free quantification based on intensity values of MS1 precursors. The relative protein abundance presented in this study represents the ratio of a protein's abundance in a specific gel slice to its overall abundance across all slices within a given BNE gel lane.

Metal analysis using inductively coupled plasma-triple quadrupole-mass spectrometry

Purified and desalted MecC and MecE proteins underwent acid digestion through incubation in 11% (v/v) HNO_3 (Suprapur) at 80 °C for 3 h. Subsequently, the samples were diluted with ultrapure water, achieving a final HNO_3 concentration of 2% (v/v). A rhodium internal standard solution was introduced to all samples, resulting in a final rhodium concentration of 1 µg L^{-1}. Calibration standards ranging between 5 ng L^{-1} and 500 µg L^{-1} were prepared by serially diluting ICP multi-element standard solution Merck XVI (Merck Millipore) in 2% (v/v) HNO_3, supplemented with the internal rhodium standard to a final concentration of 1 µg L^{-1}.

Analysis of the samples was conducted using an inductively coupled plasma-triple quadrupole-mass spectrometry system (ICP-QqQ-MS) Agilent 8800 (Agilent Technologies) in direct infusion mode with an integrated auto-sampler. The injection system comprised a Peltier-cooled (2 °C) Scott-type spray chamber with a perfluoroalkoxy alkane (PFA) nebulizer, which operated at a speed of 0.3 rps (revolutions per second), employing an internal tube diameter of 1.02 mm for 45 s. Seven metals: ^{56}Fe, ^{58}Ni,^{59}Co, ^{63}Cu, ^{66}Zn, ^{95}Mo, and ^{103}Rh, were quantified. All measurements were normalized with the internal standard. To mitigate polyatomic interferences, the Octopole Reaction System (ORS^3) with a collision/reaction cell (CRC) was used. Helium and hydrogen were introduced to the CRC as collision or reduction gases at flow rates of 2.5 mL min^{-1} and 0.5 mL min^{-1}, respectively. Argon, the carrier gas, maintained a flow rate of 2.7 mL min^{-1}. For each metal, the first (Q1) and second (Q2) quadrupoles were set to the same *m/z* value: Fe (56→56), Ni (58→58), Co (59→59), Cu (63→63), Zn (66→66), Mo (95→95), and Rh (103→103), with an integration time of 1 s under auto-detector mode. All other parameters underwent optimization through the auto-tune function in the MassHunter 4.2 operation software (Agilent Technologies).

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FIGURE LEGENDS

FIGURE 1. DCM transformation activity across blue native polyacrylamide gel electrophoresis (BNE) slices containing soluble protein fractions from the *Dehalobacterium*-containing culture. (A) Distribution of DCM transformation activity across the gel lane cut into 12 slices. The activity is represented as the percentage of DCM transformed after 24 h of incubation. Bar graphs display the mean of duplicate samples, with individual data points plotted as dots. **(B)** The relative protein abundance distribution across the gel for Mec proteins is shown as colored lines. For each protein, the relative protein abundance (%) was calculated as the ratio of the protein's abundance in a specific gel slice to its total abundance across all slices. The relative abundances for slices 1–12 sum up to 100%. Replicate results are provided in Supplementary Figure 4.

FIGURE 2. *In vitro* activity assays with recombinant MecB, MecC and MecE proteins. The assays contained different combinations of Mec proteins (5 µM each), which were incubated for 18 h with 50 µM DCM as methyl group donor. The assays were conducted with 100 mM acetate (Ac) in the reaction buffer. In some of the tests, 100 µM coenzyme M (CoM) was added as methyl group acceptor. **(A)** GC-FID chromatograms showing the DCM peak (RT ≈ 6 min) under various combinations of Mec proteins added. DCM concentrations (µM) for each condition were determined using the area under the elution peak (pA s). NEC is the non-enzyme control. **(B)** Mass spectra in the *m/z* range 58–76 of the assay solution in the negative control (NEC_{Ac}) after incubation (blue label), showing the masses for acetate (Ac, $[M\text{-}H]^-$ = 59.0134 *m/z*) detected in negative ionization mode, and **(C)** of the assay solution in the $MecEBC_{Ac}$ sample after incubation (red label), showing the mass for methyl acetate (MeOAc, $[M+H]^+$ = 75.0443 *m/z*) detected in positive ionization mode. **(D)** Mass spectra in the *m/z* range 138–160 of the assay solution in the negative control without enzyme NEC_{CoM} after incubation (orange label), showing the masses for coenzyme M (CoM, $[M\text{-}H]^-$ = 140.9682 *m/z*) detected in negative ionization mode, and methyl-CoM (MeCoM, $[M+H]^+$ = 156.9907 *m/z*) detected in positive ionization mode; as well as **(E)** of the assay solution in the $MecEBC_{CoM}$ sample after incubation (green label), showing the mass for coenzyme M (CoM, $[M\text{-}H]^-$ = 140.9684 *m/z*) detected in negative ionization mode, and methyl-CoM (MeCoM, $[M+H]^+$ = 156.9908 *m/z*) detected in positive ionization mode.

FIGURE 3. Abiotic assays reveal the interaction between reduced cob(I)alamin and dichloromethane (DCM). (A) Spectrophotometric analysis of reduced cob(I)alamin and oxidized cyanocob(III)alamin after 10 min of incubation with and without DCM. **(B)** Overlay of mass spectrometric spectra obtained after HPLC, showing the results of oxidized cyanocob(III)alamin without DCM (in black) and reduced cob(I)alamin incubated with DCM (in orange). All reaction steps were conducted under anaerobic conditions, and all masses were detected in positive ionization mode. Mass spectra in the *m/z* range 650–725 (left graph) show the masses for doubly-charged cyanocob(III)alamin (CNCbl, $[M+2H]^{2+}$ = 678.2959 *m/z*), detected in the oxidized and reduced sample lacking DCM (data not shown), as well as for chloromethyl-cob(III)alamin (MeClCbl, $[M+2H]^{2+}$ = 689.7819 *m/z*) and methyl cob(III)alamin (MeCbl, $[M+2H]^{2+}$ = 672.8055 *m/z*), detected in the reduced sample with DCM. Mass spectra in the *m/z* range 1320–1400 (right graph) show the masses for singly-charged CNCbl ($[M+H]^{+}$ = 1355.5723 *m/z*), detected in the oxidized and reduced sample without DCM (data not shown), as well as for MeClCbl ($[M+H]^{+}$ = 1378.5598 *m/z*) and MeCbl ($[M+H]^{+}$ = 1344.6003 *m/z*).

FIGURE 4. Blue native electrophoresis on purified MecB, MecC, and MecE proteins obtained through heterologous expression in *E. coli*. (A) The conformational analysis of individual MecB, MecC, and MecE proteins, each carrying a polyhistidine-tag (His-Tag), after immobilized metal affinity chromatography (IMAC) purification. Protein bands, numbered for identification, were sliced and subjected to protein mass spectrometry. A NativeMark (20–1,236 kDa, Invitrogen) served as the protein ladder. **(B)** Protein bands excised in (A) were identified using protein mass spectrometry. The absolute abundances of MS1 precursor peptides of MecB, MecC, and MecE, obtained via nLC-MS/MS Orbitrap Fusion, are presented along with the relative protein abundances (%) of the respective proteins within each gel band, calculated using MS1 intensities of the major proteins.

FIGURE 5. MecC and MecE *in silico* tertiary structures from *D. fomicoaceticum* strain EZ94 calculated using AlphaFold2. (A) Side view of superimposed MecC (pink cartoon) and MtaA from *Methanosarcina mazei* (PDB: 4AY7, grey cartoon) and the zinc atom of MtaA, located in the center of the TIM-barrel structure (dark grey sphere). The root-mean-square deviation (RMSD) between the two protein structures was 2.2 Å. The funnel is indicated with an arrow **(B)** Top view of MecC (pink cartoon) highlighting the amino acid

residues (yellow sticks) predicted to bind three zinc atoms (Zn1–Zn3) in the periphery. The putative zinc atoms (red spheres) are positioned close to the predicted amino acids, with distances mostly <3 Å. **(C)** Side view of MecE (green cartoon) aligned with MtaA from *M. mazei* (grey cartoon), with the zinc atom of MtaA in the middle of the TIM-barrel (dark grey sphere). The RMSD calculated between the two proteins was 1.6 Å. The funnel is indicated with an arrow **(D)** Top view of MecE (green cartoon) with the highly conserved amino acid residues (Cys193, His225, Cys227, and Cys312) (yellow sticks), predicted to coordinate zinc (red spheres), with the distance <3 Å, in the middle of the protein structure.

FIGURE 6. *In silico* tertiary structure of MecBE heterodimer calculated with AlphaFold2. A side view of MecB (cyan) and MecE (green) forming a heterodimer. In the Rossmanoid fold domain of MecB, the histidine residue His103 (blue stick representation) coordinates cobalamin (purple stick representation). The predicted and conserved zinc binding site is located in the funnel of the TIM-barrel-like structure of MecE. The zinc ion in MecE (red sphere) is most probably coordinated by highly conserved amino acid residues Cys193, His225, Cys227 and Cys312, with distances <3 Å.

FIGURE 7. Proposed proteins involved in the initial steps of dichloromethane transformation in the metabolism of *Dehalobacterium formicoaceticum* strain EZ94 based on the results of our study.