The 380 kb pCMU01 Plasmid Encodes Chloromethane Utilization Genes and Redundant Genes for Vitamin B\textsubscript{12}- and Tetrahydrofolate-Dependent Chloromethane Metabolism in Methylobacterium extorquens CM4: A Proteomic and Bioinformatics Study

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Abstract

Chloromethane (CH\textsubscript{3}Cl) is the most abundant volatile halocarbon in the atmosphere and contributes to the destruction of stratospheric ozone. The only known pathway for bacterial chloromethane utilization (cmu) was characterized in Methylobacterium extorquens CM4, a methylotrophic bacterium able to utilize compounds without carbon-carbon bonds such as methanol and chloromethane as the sole carbon source for growth. Previous work demonstrated that tetrahydrofolate and vitamin B\textsubscript{12} are essential cofactors of cmuA- and cmuB-encoded methyltransferases of chloromethane dehalogenase, and that the pathway for chloromethane utilization is distinct from that for methanol. This work reports genomic and proteomic data demonstrating that cognate cmu genes are located on the 380 kb pCMU01 plasmid, which drives the previously defined pathway for tetrahydrofolate-mediated chloromethane dehalogenation. Comparison of complete genome sequences of strain CM4 and that of four other M. extorquens strains unable to grow with chloromethane showed that plasmid pCMU01 harbors unique genes without homologs in the compared genomes (blub2, btuB, cobA, cbID), as well as 13 duplicated genes with homologs of chromosome-borne genes involved in vitamin B\textsubscript{12}-associated biosynthesis and transport, or in tetrahydrofolate-dependent metabolism (folC2). In addition, the presence of both chromosomal and plasmid-borne genes for corrinoid salvaging pathways may ensure corrinoid coenzyme supply in challenging environments. Proteomes of M. extorquens CM4 grown with one-carbon substrates chloromethane and methanol were compared. Of the 49 proteins with differential abundance identified, only five (CmuA, CmuB, PurU, CobH2 and a PaaE-like uncharacterized putative oxidoreductase) are encoded by the pCMU01 plasmid. The mainly chromosome-encoded response to chloromethane involves gene clusters associated with oxidative stress, production of reducing equivalents (PntAA, Nuo complex), conversion of tetrahydrofolate-bound one-carbon units, and central metabolism. The mosaic organization of plasmid pCMU01 and the clustering of genes coding for dehalogenase enzymes and for biosynthesis of associated cofactors suggests a history of gene acquisition related to chloromethane utilization.

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Introduction

Chloromethane (CH\textsubscript{3}Cl) is a volatile organic compound emitted by oceans, plants, wood-rotting fungi and biomass burning, estimated to account for 17% of chlorine-catalyzed ozone degradation in the stratosphere [1]. Chloromethane-utilizing bacteria have been isolated from a wide variety of environments such as seawater, soil, sludge, and recently from plant leaf surfaces [2], and represent a potential biotic filter for chloromethane emissions. Many chloromethane degraders are facultative methylotrophic Proteobacteria [3] growing in aerobic metabolism with chloromethane and other C\textsubscript{1} carbons such as methanol as unique source of carbon and energy. Complete and assembled genomes of two chloromethane-utilizing strains, Methylobacterium extorquens strain CM4 and Hyphomicrobium sp. strain MC1, are available [4,5]. The only known microbial aerobic utilization pathway for chloromethane is tetrahydrofolate (H\textsubscript{4}F)-dependent [6]. This pathway was identified in the alpha-Proteobacterium M. extorquens CM4 using mini-transposon random mutagenesis [7] and its chloromethane dehalogenase activity characterized in detail [8,9]. The first step of the cmu (chloromethane utilization) pathway is catalyzed by the two-domain methyltransferase/corrinoid-binding CmuA protein that transfers the methyl group from chloromethane to a corrinoid cofactor [9,10]. The methylcobalamin:H\textsubscript{4}F methyltransferase CmuB enzyme subsequently catalyzes the transfer of the methyl group from the corrinoid cofactor to H\textsubscript{4}F [9]. The H\textsubscript{4}F-bound C\textsubscript{1}
moiety of chloromethane, methylene-\(\text{H}_4\text{F}\) (\(\text{CH}_2 = \text{H}_4\text{F}\)) is oxidized to carbon dioxide via formate to produce energy, or funneled into the serine pathway for biomass synthesis (Fig. 1). Evidence that \(\text{H}_4\text{F}\) is an essential cofactor of the \(\text{cmu}\)-dependent degradation of chloromethane was obtained from mutant analyses in \(M. \text{extorquens}\) CM4, which identified \(\text{metF}\) (encoding methylene-\(\text{H}_4\text{F}\) reductase) and \(\text{purU}\) (encoding formyl-\(\text{H}_4\text{F}\) hydrolase) as essential genes for growth with chloromethane [10]. The pathway for chloromethane utilization in \(Methyllobacterium\) is thus completely different from that for dichloromethane \((\text{CH}_2\text{Cl}_2)\), which involves \(\text{DcmA}\), a cytoplasmic dichloromethane dehalogenase/glutathione S-transferase yielding the central intermediate of methylotrophic metabolism formaldehyde \((\text{HCHO})\) [11].

Figure 1. Methylotrophic metabolism and chloromethane utilization pathway in \(Methyllobacterium \text{extorquens}\) CM4. The left-hand scale indicates carbon oxidation state. The chloromethane utilization \(\text{cmu}\) pathway (bold arrows) funnels the chloromethane-derived methyl group into central metabolism via methylene-\(\text{H}_4\text{F}\) (\(\text{CH}_2 = \text{H}_4\text{F}\)), while the methanol \((\text{CH}_3\text{OH})\) oxidation pathway operates with formaldehyde \((\text{HCHO})\) as a metabolic intermediate (grey arrows). \(\text{H}_4\text{F}\) - and \(\text{H}_4\text{MPT}\)-dependent enzyme-mediated steps are depicted in blue and pink, respectively. Carbon assimilation operates via the serine cycle \((\text{Ser})\) coupled with the ethylmalonyl-CoA pathway \((\text{EMCP})\) [67]. Spontaneous condensation of \(\text{HCHO}\) with \(\text{H}_4\text{F}\) or \(\text{H}_4\text{MPT}\), and formaldehyde oxidation to methylene-\(\text{H}_4\text{F}\) are shown with broken line. In the \(\text{cmu}\) pathway, the methyl group enters a specific \(\text{H}_4\text{F}\)-oxidation pathway for energy production driven by the \(\text{ FolD}\) and \(\text{ PurU}\) enzymes. Protein-encoded genes or genes located on plasmid pCMU01 are shown in bold. Boxes and circles highlight proteins more abundant in chloromethane- and methanol grown-cultures, respectively. \(\text{CmuB}\), methytransferase/corrinoid-binding two-domain protein; \(\text{CmuB}\), methylcobalamin:\(\text{H}_4\text{F}\) methyltransferase; \(\text{Fae}\), formaldehyde activating enzyme; \(\text{Fch}\), methylen-\(\text{H}_4\text{F}\) cyclohydrodase; \(\text{FDHs}\), formate dehydrogenases; \(\text{Fhc}\), formyltransferase-hydrodase complex; \(\text{FolD}\), bifunctional methylene-\(\text{H}_4\text{F}\) dehydrogenase/cyclohydrodase; \(\text{Ftl}\), formate-\(\text{H}_4\text{F}\) ligase; \(\text{Gck}\), glycerate kinase; \(\text{GcvT}\), \(\text{H}_4\text{F}\)-dependent aminomethyltransferase; \(\text{HprA}\), hydroxypropionate reductase; \(\text{MDH}\), methanol dehydrogenase; \(\text{MetF}\), methylen-\(\text{H}_4\text{F}\) reductase; \(\text{MtdA}\), bifunctional \(\text{NAD(P)}\)-dependant methylene-\(\text{H}_4\text{F}\) and methylene-\(\text{H}_4\text{MPT}\) dehydrogenase; \(\text{MtdB}\), \(\text{NAD(P)}\)-dependant methylene-\(\text{H}_4\text{MPT}\) dehydrogenase; \(\text{Mch}\), methylen-\(\text{H}_4\text{MPT}\) cyclohydrodase; \(\text{MtkA}\), malate thikinase large subunit; \(\text{MxaF}\), \(\text{MDH}\) alpha subunit, \(\text{PurU}\), 10-formyl-\(\text{H}_4\text{F}\) hydrolase; \(\text{Sga}\), serine-glyoxylate aminotransferase [12]. Plasmid pCMU01 encoded proteins with predicted functions include putative uncharacterized methyltransferases \(\text{CmuC}\) and \(\text{CmuC2}\), the putative \(\text{PaaE}\)-like oxidoreductase, and the putative \(\text{PQQ}\)-linked dehydrogenase of unknown specificity \(\text{XoxF2}\). \(\text{GcvT}\) may serve to transfer methyl groups from a wide range of substrates to \(\text{H}_4\text{F}\), as proposed for members that belong to the \(\text{COG0354}\)-related enzymes such as \(\text{YgfZ}\) [68].

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Known pathways for tetrahydromethanopterin (H₄MPT)- and H₄F-dependent C₁ substrate oxidation in Methylobacterium strains are compared in Figure 1. When growing on methanol, M. extorquens CM4 uses the H₄MPT formaldehyde oxidation pathway first discovered in M. extorquens AM1 [12] and subsequently found to be widespread among methylo trophs.

Growth with chloromethane depends on the presence of cobalt in the medium [9] since CmuA methyltransferase activity requires a vitamin B₁₂-related corrinoid cofactor that incorporates cobalt. As described for adenosylcobalamin (AdoCbl), the corrinoid cofactor may be synthesized de novo by one of Nature’s most complex metabolic pathways requiring around 30 enzyme-mediated steps [13,14]. Of those, only cobUQD genes found adjacent to cmu genes have been described in M. extorquens CM4 [10]. Many microorganisms synthesize vitamin B₁₂-related compounds from imported corrinoid intermediates [14] or from precursors such as dimethylbenzimidazole (DMB) [15] by pathways that have not been identified in chloromethane-degrading bacteria.

In this work, combined experimental and bioinformatics analysis was performed to gain a better understanding of the genes and proteins specifically associated with chloromethane utilization in M. extorquens CM4. A differential proteomics approach compared M. extorquens CM4 proteins under methylotrophic growth conditions with either chloromethane or methanol as the sole carbon and energy source. Gene clusters specific to the chloromethane response were identified, and compared to previously published clusters involved in the response of M. extorquens DM4 to dichloromethane [16], or involved in the methylothrophic growth of M. extorquens AM1 to methanol [17]. We found that growth with chloromethane elicits a specific adaptive response in M. extorquens CM4. In addition, the genome sequence of the chloromethane-degrading strain CM4 was compared to available complete sequences of other M. extorquens strains unable to grow on chloromethane [strains AM1, PA1, BJ001 and DM4; [5,11]]. Genomic analysis revealed that additional gene homologs of chromosome-encoded cognate genes for coenzyme biosynthesis, as well as specific genes such as bluB2, which is predicted to be involved in both H₄F and vitamin B₁₂ cofactor biosynthesis, were found nearby characterized genes cmuA and cmuB on a 380 kb plasmid.

Materials and Methods

Manual Gene Annotation and Bioinformatic Analysis

Comparative analyses were performed using the fully sequenced genomes of four representatives of the M. extorquens species; strain CM4 (GenBank accession no. CP001298, CP001299, CP001300), strain AM1 (GenBank accession no. CP001511, CP001512, CP001513 and CP001514), strain DM4 (GenBank accession no. FP103042 and FP103043 and FP103044), strain PA1 (GenBank accession no. CP000098) and strain BJ001 (GenBank accession no. CP001029, CP001030 and CP001031) [5]. Putative orthology relationships were operationally defined by gene pairs from different genomes satisfying an alignment threshold of at least 40% amino acid sequence identity (aa Id) over at least 80% of the length of the smallest encoded protein. The search for conserved genes clusters was performed as previously described [11]. Manual validation of automatic gene annotations on pCMU01 plasmid (also known as pCMU01) was performed using the relational database [18] Microscope web interface (MethyloScope) https://www.genoscope.cns.fr/agc/mage/wwwpkgdb/Login/log.php?pid=26; Insertion sequence (IS) annotations were done as previously described [11]. IS elements were given names of type “ISMch3”, with “Mch” for Methylobacterium extorquens degrading chloromethane.

Biological Materials, Media and Growth Conditions

The composition of Methylobacterium mineral medium M3 was adapted from that given in Vannelli et al. [7] with 0.2 g L⁻¹ (NH₄)₂SO₄ final concentration and substitution of ZnCl₂ by ZnSO₄ in the trace element solution. M. extorquens CM4 was grown aerobically at 30°C either with chloromethane or with methanol as carbon substrate, on a rotary shaker (140 rpm) in 1.2-liter Erlenmeyer flasks containing 200 mL. M3 medium, closed with gas-tight screw caps with mininert valves (Supelco). Methanol (sterile-filtered) was added to a final concentration of 40 mM. Chloromethane gas was added to a final concentration of 15 mM in the liquid phase, assuming a Henry constant of 0.0106 m³ atm⁻¹ mol⁻¹ at 30°C [19], as previously described [6]. Acetone was added to a final concentration of 5 mM from a sterile-filtered solution at 250 mM. Chloromethane and acetone degradation were quantified using a CP 3900 gas chromatograph connected to a flame ionization detector (GC-FID; Varian, USA) equipped with a GC column (CP-Sil 5 CB, length 15 m; Varian).

Protein Extraction

Triplicates of M. extorquens CM4 cultures were harvested by centrifugation (10 min at 10,000 g) in mid exponential growth phase of chloromethane- and methanol-grown cultures, using 100 mL at OD₆₀₀ of 0.2 and 33 mL at OD₆₀₀ at 0.6, respectively. Cell pellets were resuspended in 10 mM Tris, 1 mM EDTA buffer pH 7.6 (TE buffer), washed once and resuspended in 400 mL of the same buffer in the presence of benzolase (250 units; GE Healthcare) and 4 μL protease inhibitor mix 100× (GE Healthcare). Cells were disrupted using glass beads (0.1 mm in diameter, 1 g per 0.4 mL extract) in a MM2 mixer mill (Retsch Haan, Germany) at maximal speed for 6 cycles of 30 sec, and then placed on ice for one hour. Cell debris and beads were removed by centrifugation at 14,000 g for 15 min at 4°C, and the supernatant was centrifuged again at 14,000 g for one hour at 4°C. Protein concentration in the supernatant was assayed using a commercial Bradford assay (Biorad) with bovine serum albumin as a standard, and subsequently adjusted to 1 mg/mL in TE buffer.

Two-dimensional Gel Electrophoresis (2D–E)

Protein extracts (100 μg) were precipitated overnight with 9 vol. of acetone at 4°C, centrifuged at 10,000 g for 10 min at 4°C, washed three times with 80% acetone. Proteins were resuspended in 350 μL of purified rehydration buffer (RB); BB buffer (7 M urea, 2 M thiouria) was purified by mixing for one hour with 10 g L⁻¹ Amberlite IRN-150L (GE Healthcare), 2.5% wt/vol CHAPS (3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonate], 0.6% immobilized pH gradient (IPG) buffer (pH range 4–7 or 3–10, respectively), 65 mM dithiothreitol (DTT), and 0.002% wt/vol bromophenol blue. Proteins (50 μg) were loaded on 18-cm IPG strips (linear gradient pH 4–7 and 3–10) with the IPGphor 3 isoelectric focusing (IEF) system, as recommended by the manufacturer (GE Healthcare). Rehydration (6 h at 0 V and 6 h at 30 V) was followed by four two-hour increments during which the voltage was increased stepwise from 150 V, 500 V, 1000 V, to 3000 V. Finally, separation was obtained using 8000 V until a minimum of 45,000 Vh⁻¹ was reached, and strips were stored at −80°C. Before use, strips were thawed at room temperature, and placed in equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue) twice for 15 min, first with 10 mg/mL DTT and then with 25 mg/mL Iodoacetamide. The second dimension
of electrophoretic separation was performed by 11.5% SDS-PAGE using the Ettan DALTII system (GE Healthcare). Gels were fixed during 1 h in a 40% ethanol, 7% acetic acid solutions. Proteins were stained overnight with Brilliant Blue G-collodial (Sigma). Scanning of gels was performed on an Image Scanner (GE Healthcare) with LabScan software (GE Healthcare).

Two-dimensional Fluorescence Differential Gel Electrophoresis (2D-DIGE)

Samples were labeled with CyDye DIGE Fluor minimal dyes (Cy2, Cy3 or Cy5, GE Healthcare) according to the manufacturer’s instructions. After acetone precipitation, the resulting protein pellet was resuspended in DIGE rehydration buffer (DRB) (7 M urea, 2 M thiourea, 4% wt/vol CHAPS, Tris 20 mM, pH 8.5). The protein concentration was quantified using a slightly modified Bradford method (as described above except for use of DRB), standardized with known concentrations of bovine serum albumin, and adjusted to 2 mg/mL using DRB. For each DIGE experiment, 8 samples were labeled, corresponding to four independent cultures for each condition. Labeling was performed by mixing 50 µg total protein from each of the four samples with either Cy3 (two samples) or Cy5 (two other samples) DIGE minimal dye (400 pmol) (GE Healthcare), to take into account biases resulting from different labeling efficiency. A pooled set of internal standards, comprising 25 µg aliquots from each of the 8 samples (200 µg total), was labeled with Cy2 DIGE minimal dye (1600 pmol total). Labeling was performed for 30 minutes on ice and in the dark, and quenched by the addition of 10 mM lysine (1 µL for Cy3- or Cy5-labeled extracts, and 4 µL for Cy2-labeled extracts, respectively). Samples were incubated for 10 min on ice in the dark. Finally, protein samples that were separated on the same gel were mixed (one Cy3- and one Cy5-labeled sample each together with one-fourth volume of the pooled set of internal standards), and supplemented with 0.6% IPG pH 3–10 NL (non-linear) and 65 mM DTT. Buffer RB was added to each mix to final volume of 350 µL before IEF separation. Proteins were loaded on 18-cm IPG strips (non-linear gradient pH 3–10) and submitted to separation steps as described above. Gels were fixed and proteins stained as described above. Scanning of gels was performed with an Ettan DIGE Imager (GE Healthcare).

Proteome Image Analysis

Differential analysis was performed using ImageMaster 2D Platinum software (v. 6.0, GE Healthcare). Six gels were grouped in two classes of three independent gels depending on the two compared conditions (chloromethane or methanol growth conditions). Gels were matched with one reference gel (master gel) following spot detection. For each spot, the relative volume corresponded to the normalized volume of the spot compared to the normalized volume of the entire gel coloration. Statistical analysis was performed by calculating the Student t value for each spot, as well as a ratio value defined as the mean of the relative volume of the spot obtained in the different replicates for growth with chloromethane divided by the mean of the relative volumes obtained for growth with methanol. Spots with a Student t value higher than 1.9 (corresponding to a p-value of <0.1) and ratios ≥2.0 or ≤2.0 were analyzed by mass spectrometry. DIGE images were analyzed with DeCyder software (v. 7.0, GE Healthcare). A total of twelve images obtained from 4 gels (three images each) were analyzed. Student’s t test was used to determine differential abundance of proteins. In this procedure, the p-values were corrected for false discovery rate [20]. Spots with a p-value <0.01 and ratios ≥2.0 or ≤2.0 were considered to be differentially abundant.

Mass Spectrometry Protein Identification

The procedure of Muller et al. [16] was followed for spot identification, with minor adjustments. Mass spectrometry analyses were performed in reflector positive mode on a Biflex III (Bruker-Daltonik GmbH, Bremen, Germany) matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF TOF) and on an Autoflex III Smartbeam (Bruker-Daltonik GmbH, Bremen, Germany) matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF TOF). A saturated solution of α-cyano-4-hydroxybutyric acid in 50% water/50% acetonitrile was used as matrix for MALDI mass measurement on the Biflex III. Pepsin mass fingerprinting data (PMF) and peptide fragment fingerprinting data (PFF) were combined by Biotools 3 software (Bruker Daltonik) and transferred to the search engine MASCOT (Matrix Science, London, UK). Pepsin mass error was limited to 0.1 ppm for the Biflex III and to 50 ppm for the Autoflex III Smartbeam. Proteins were identified by searching against the NCBI non-redundant protein sequence database and the predicted proteins of strain CM4 (GenBank accession no. CP001298, CP001299, CP001300).

Results

The 380 kb Episome in M. extorquens CM4 Harbors cmu Genes and Associated Genes

The repertoire of known cmu genes and genes conserved in chloromethane-degrading strains includes genes essential for dehalogenation of chloromethane (cmuA, cmuB, cmuC), genes essential for growth with chloromethane as the sole carbon and energy source (cmuC, metF, pufU), and genes found in the vicinity of genes cmuA, cmuB and cmuC in methylo trophic chloromethane-degrading strains (findB, paeE, hutL, and cbdB) [2]. All these genes co-localize on a 138 kb region flanked by transposable elements nested within the 380 kb plasmid pCMU01 in M. extorquens CM4. This plasmid encodes proteins associated with growth on chloromethane, such as the enzymes for chloromethane degradation and for metabolism of the two essential dehalogenase cofactors AdoCbl and H2F, as well as transport proteins for coenzyme B12 precursors (Table 1).

Thus, plasmid pCMU01 can be designated as a chloromethane catabolic plasmid that harbors the cognate essential genes for growth on chloromethane.

Overview of Plasmid pCMU01 Gene Content

Plasmid pCMU01 is characterized by a somewhat lower GC content (66.3%) than the chromosome of strain CM4 (68.2%). To a large extent (41%), it features unique genes encoding predicted proteins without close homologs (>40% aa Id, >80% of the protein length) in the genomes of four other M. extorquens strains unable to degrade chloromethane [5] (Table 1). Of its 386 predicted CDS, 56% belong to at least one COG group [21] related to metabolism (enzymes, 69 CDS; transporters, 20 CDS), plasmid functions (23 CDS), adaptive response (regulation, 34 CDS; stress, two CDS), and genomic plasticity (mobile DNA elements, 71 CDS with a total of 18 identified IS elements representing 4% of the predicted CDS of the plasmid). Pseudo genes may account for 9% of the total predicted CDS on the plasmid with 33 pseudogenes detected.

Chloromethane is not the only organic molecule for which the plasmid allows to transform for growth. A complete acetone-catabolic gene cluster encoding the acetone carboxylase subunits (β subunit, acxA; α subunit, acxB; γ subunit, acxC) and its cognate transcriptional activator (gene acxR) was found. Acetone carboxylase is the key enzyme of bacterial acetone metabolism in Xanthobacter autotrophicus strain Py2, catalyzing the ATP-dependent
carboxylation of acetone to form acetoacetate [22]. High sequence conservation was found between the acsRABC gene clusters of strains CM4 and X. autotrophicus Py2 (>82% aa Id for enzyme subunits and 53% for the regulator). The ability of M. extorquens CM4 to degrade acetone was tested in aerobic liquid cultures in M. extorquens subunits and 53% for the regulator). The ability of repABC similarity searches. A combined replication and partitioning conjugation of plasmid pCMU01 was suggested from sequence conservation was found between the M. extorquens genomes including that of strain CM6. Occasional, 173 pCMU01 plasmid-encoded proteins with homologs in at least one of the S. M. extorquens genomes. Plasmid pCMU01 and plasmid p1METDI of strain DM4 share 56 homologs localized on three gene clusters. Selected examples are indicated when relevant.

pCMU01 plasmid encoded protein MetF2 (Mchl_5726) previously demonstrated to be essential for chloromethane utilization [6] encodes a protein with only 25% aa Id to E. coli MetF. It is more distantly related to the canonical MetF than its chromosomal homolog (Mchl_1881, 56% aa Id to E. coli MetF).

UTrA universal stress protein (Mchl_5472) also found in the DCM-dehalogenating M. extorquens DM4 only (METDI4473). Close homologs (70% Id aa) located in synteny on the 1.26 Mb megaplasmid of strain AM1.

Table 1. Analysis of the theoretical proteome of plasmid pCMU01.

| Functional class | Occurrence in sequenced Methylbacterium extorquens genomes* |
|-----------------|-------------------------------------------------------------|
|                 | Unique | Common | Occasional |                               |
| Chloromethane degradation | 6 (CmuA, CmuB, CmuC, CmuC2, Hutl, PaaE-like) | 0 | 0 |
| Cobalamin metabolism | 3 (BtuB2, CbiD, CobA) | 13 (Cob protein) | 0 |
| H2F and C1 metabolism | 3 (FolD, MetF2, PurU) | 4 (FolC2, Hss2, SerC2, XoxF2) | 0 |
| Acetone degradation | 3 (AcxA, AcxB, AcxC) | 0 | 0 |
| Other metabolisms | 7 | 10 (Gck2, Shc2) | 19 (GdhA, IspF, SorA, SorB) |
| Stress | 0 | 1 (UspA fragment) | 1 (Usp) |
| Plasmid-related function | 3 (MmeI) | 1 (ArDc) | 19 (DotABC-like, IcmBCEKL, TraGDCA, RepABC) |
| Transporter | 3 | 10 (BtuC, BtuF, BtuD, CldA, ModA2, ModB2, ModC2, Modp2) | 7 (BtuB, CzcBA2) |
| Regulator | 14 (AcxR, FmdB) | 8 | 12 (CzcSR) |
| Mobile element-related | 25 | 3 | |
| Unknown | 90 | 6 | 72 |

*Compared predicted proteome sizes are, M. extorquens strains AM1, 6531 proteins (genome sequence accession no NC_012806); DM4, 5773 proteins (NC_012988); PA1, 5357 proteins (NC_010171); CM4, 4654 proteins (NC_011757); BJI01, 6027 proteins (NC_010725). Homologous proteins were defined as proteins with at least 40% identity covering over 80% of the sequence. Three classes of proteins were considered: Unique, 157 pCMU01 plasmid-encoded proteins without homologs in any of the compared genomes, including the chromosome and the second plasmid p2MCHL of strain CM4; Common, 56 pCMU01 plasmid-encoded proteins with homologs on the chromosome of all 5 M. extorquens genomes; Occasional, 173 pCMU01 plasmid-encoded proteins with homologs in at least one of the 5 M. extorquens genomes. Plasmid pCMU01 and plasmid p1METDI of strain DM4 share 56 homologs localized on three gene clusters. Selected examples are indicated when relevant.

**pCMU01 plasmid encoded protein MetF2 (Mchl_5726) previously demonstrated to be essential for chloromethane utilization [6] encodes a protein with only 25% aa Id to E. coli MetF. It is more distantly related to the canonical MetF than its chromosomal homolog (Mchl_1881, 56% aa Id to E. coli MetF).**

**UTrA universal stress protein (Mchl_5472) also found in the DCM-dehalogenating M. extorquens DM4 only (METDI4473).**

**Close homologs (70% Id aa) located in synteny on the 1.26 Mb megaplasmid of strain AM1.**

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Extensive Plasmid-encoded Gene Redundancy Associated with Vitamin B12 Metabolism

M. extorquens CM4 is able to synthesize coenzyme B12, a cofactor essential for activity of chloromethane dehalogenase CmuAB [9]. A complete set of cob genes homologous to those described in P. denitrificans for the aerobic biosynthesis pathway of AdoCbl [14] is found on the chromosome of CM4 as well as on the chromosomes of four other M. extorquens strains (Table 2). Remarkably, strain components of a core type IVB secretion/conjugation system complex often used for horizontal propagation, including the gene encoding the conserved central component of the DNA transport activity core complex (Mchl_5595), and traD (Mchl_5572) which lies within a traGDCA gene cluster (Mchl_5572–5575) conserved in other Alphaproteobacteria plasmids including Agrobacterium tumefaciens T plasmids (encoded proteases TraG, D, C and A sharing 43, 53, 38 and 44% aa Id, respectively). Finally, a putative restriction-modification system encoding protein Mchl_5634 shares 48% aa Id with a bifunctional DNA methyltransferase/type II restriction endonuclease MmI [26] (Table 1). Taken together, the described genomic features indicate that plasmid pCMU01 represents a low-copy plasmid, vertically transmitted via a RepABC replication and partitioning unit, and most probably able to propagate by horizontal transfer within Alphaproteobacteria.
Table 2. Gene redundancy for cobalamin and tetrahydrofolate metabolism in *M. extorquens* CM4.

| Function                  | Gene in strain CM4 | Occurrence in *M. extorquens* | MaGe annotation | EC n° | Plasmid pCMU01 identifier |
|---------------------------|--------------------|--------------------------------|-----------------|-------|---------------------------|
|                           | Chromosome         | Plasmid pCMU01 | Paralog Id (%) |       |                           |
| Cobalamin metabolism      |                    |                 |                |       |                           |
| Aerobic AdoCbl biosynthesis from precursors |                    |                 |                |       |                           |
|                           | bluB               | bluB2           | 38.4           | core  | 5,6-dimethylbenzimidazole synthase (flavin destructase), putative cob(I)yrinic acid a,c-diamide reductase | 1.16.8.1 | Mchl_5732 |
|                           | /                  | cbID            | /              | CM4 specific | Cobalamin biosynthesis protein, putative cobalt-precorrin-6A synthase [deacetylating] | 2.1.1.- | Mchl_5729 |
|                           | /                  | cobA a          | /              | CM4 specific | S-adenosyl-L-methionine-dependent uroporphyrinogen III methylase (SUMT) | 2.1.1.107 | Mchl_5731 |
|                           | cobB               | /               | /              | core    | Cob(II)yrinic acid a,c-diamide synthase | / |
|                           | cobC               | cobC2 a         | 51.2           | core    | L-threonyl-O-3-phosphate decarboxylase | 4.1.1.81 | Mchl_5730 |
|                           | cobD               | cobD2           | 76.4           | core    | Cobalamin biosynthesis protein CobD | Mchl_5724 |
|                           | cobE               | cobE2           | 64.4           | core    | Cobalamin biosynthesis protein CobE | Mchl_5686 |
|                           | cobF               | /               | /              | core    | Putative precorrin-6A synthase | 2.1.1.152 | / |
|                           | cobG               | /               | /              | core    | Putative precorrin-3B synthase CobG | 1.14.13.83 | / |
|                           | cobH               | cobH2           | 86.2           | core    | Precorrin-8X methyltransferase | 5.4.1.2 | Mchl_5691 |
|                           | cobI               | cobI2           | 80.7           | core    | Precorrin-2 C(20)-methyltransferase | 2.1.1.130 | Mchl_5690 |
|                           | cobJ               | cobJ2           | 79.1           | core    | Precorrin-3B C(17)-methyltransferase | 2.1.1.131 | Mchl_5689 |
|                           | cobK               | cobK2           | 63.4           | core    | Precorrin-6A reductase | 1.3.1.54 | Mchl_5688 |
|                           | cobL               | cobL2           | 76.0           | core    | Precorrin-6Y C(5,15)-methyltransferase | 2.1.1.132 | Mchl_5687 |
|                           | cobM               | cobM2           | 82.7           | core    | Precorrin-4 C(11)-methyltransferase | 2.1.1.133 | Mchl_5685 |
|                           | cobN               | /               | /              | core    | Putative cobaltochelatase, CobN-related | 6.6.1.2 | / |
|                           | cobO               | cobO2           | 83.5           | core    | Cob(I)yrinic acid a,c-diamide adenosyltransferase | 2.5.1.17 | Mchl_5722 |
|                           | cobP               | cobP2           | 73.6           | core    | Bifunctional adenosylcobalamin biosynthesis protein CobP | 2.7.7.62 | Mchl_5721 |
|                           | cobQ               | cobQ2           | 75.2           | core    | Cobyrinic acid synthase | Mchl_5723 |
|                           | cobS               | /               | /              | core    | Aerobic cobaltochelatase subunit CobS | 6.6.1.2 | / |
|                           | cobT               | /               | /              | core    | Aerobic cobaltochelatase subunit CobT | 6.6.1.2 | / |
|                           | cobU               | cobU2           | 54.5           | core    | Nicotinate nucleotide–dimethylbenzimidazole phosphoribosyltransferase | 2.4.2.21 | Mchl_5702 |
|                           | cobV               | /               | /              | core    | Cobalamin synthase | 2.1.1.1 | / |
|                           | cobW               | /               | /              | core    | Cobalamin biosynthesis protein CobW | / |
|                           | cobW               | /               | /              | core    | Putative cobalamin biosynthesis protein CobW | / |
|                           | cobW               | /               | /              | core    | Putative cobalamin biosynthesis protein CobW | / |
|                           | cysG               | /               | /              | core    | Siroheme synthase | 2.1.1.107/13.1.76/499.1.4 | / |
Table 2. Cont.

| Gene in strain CM4 | Paralog in strain CM4 | occurrence in M. extorquens | Chromosome | Plasmid pCMU01 | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|-------------------|----------------------|-----------------------------|------------|----------------|-----------------------------------------------|
| Function          | Gene                  | Occurrence in M. extorquens | Barcoding   | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquens | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquen | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquen | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquen | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquen | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquen | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | GenBank     | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metabolism |
|                   |                      |                             | M. extorquen | pCMU01 Plasmid | pCMU01 Plasmid-Driven Chloromethane Metab
| Function | Gene in strain CM4 | Occurrence in M. extorquens<sup>a</sup> | MaGe annotation<sup>b</sup> | EC n° | Plasmid pCMU01 identifier |
|----------|-------------------|----------------------------------------|-----------------------------|--------|---------------------------|
|          |                   | Parallel aa Id (%)                   |                             |        |                           |
| Chromosome | Plasmid pCMU01 |                                  |                             |        |                           |
| pabB     | /                 | core                                  | Para-aminobenzoate synthase component I | 26.1.85 | /                         |
| pabC     | /                 | core                                  | Putative 4-aminob-4-deoxychorismate lyase component of para-aminobenzoate synthase | 41.3.38 | /                         |
| D-erythrose-4P to chorismate |       |                                          |                             |        |                           |
| aroA     | /                 | core                                  | 3-enolpyruvyshikimate-5-phosphate synthetase | 25.1.19 | /                         |
| aroC     | /                 | core                                  | Chlorismate synthase        | 42.3.5 | /                         |
| aroE     | /                 | core                                  | Putative shikimate 5-dehydrogenase | 11.1.25 | /                         |
| aroG     | /                 | core                                  | 2-dehydro-3-deoxyphosphoheptonate aldolase | 4.1.254 | /                         |
| aroK     | /                 | core                                  | Putative transcriptional regulator (N-terminal)/shikimate kinase (C-terminal) | 2.7.1.71 | /                         |
| aroQ     | /                 | core                                  | 3-dehydroquinol dehydratase, type II | 42.1.10 | /                         |
| Mchl_1923 | /                 | core                                  | Bifunctional shikimate kinase (AroK)/dehydroquinol synthase (AroB) | 42.3.4 | /                         |
| D-erythrose-4P synthesis from sugars |       |                                          |                             |        |                           |
| cbbA     | /                 | core                                  | Fructose-bisphosphate aldolase | 4.1.23 | /                         |
| fbp      | /                 | core                                  | Fructose-1,6-bisphosphatase I | 3.1.11 | /                         |
| glpX     | /                 | core                                  | Fructose 1,6-bisphosphatase II | 3.1.11 | /                         |
| tpiA     | /                 | core                                  | Triosephosphate isomerase   | 5.3.1 | /                         |
| Tetrahydrofolate interconversion |       |                                          |                             |        |                           |
| /        | folD              | CM4 specific                           | Bifunctional methylene-H<sub>4</sub>F dehydrogenase/methenyl-H<sub>4</sub>F cyclohydrodralase | 1.5.1.5/3.5.4.9 | Mchl_5700 |
| /        | fthL              | core                                  | Formate-H<sub>4</sub>F ligase | 63.43 | /                         |
| /        | gcvH              | core                                  | Glycine cleavage complex protein H | /      | /                         |
| /        | gcvP              | core                                  | Glycine cleavage complex protein L, (dihydrolipoamide dehydrogenase) | 18.1.4 | /                         |
| /        | gcvT              | core                                  | Glycine cleavage complex protein P, PLP-dependent glycine dehydrogenase | 14.42 | /                         |
| /        | glyA              | core                                  | Serine hydroxymethyltransferase | 2.1.120 | /                         |
| /        | metF              | 26                                    | 5,10-methylene-H<sub>4</sub>F reductase | 15.1.20 | Mchl_5726 |
| /        | purH              | core                                  | Bifunctional IMP cyclohydrodralase/phosphoribosyl-aminomimidazolecarboxamide formyltransferase | 35.4.10/21.23 | /                         |
| /        | purN              | purU<sup>+</sup>                       | Phosphoribosyl/glycinamid formyltransferase | 21.2.23 | Mchl_5699 |

<sup>a</sup>Homologs with ≥90% aa Id (with mentioned exceptions) found in the chromosome of all M. extorquens strains AM1, BJ001, DMA, and PA1 (common core genome), in one of the strains (shared accessory genome), or none of these strains (CM4 specific CDS). The accessory genome includes a btuB homolog (Mpop_3807, 65% aa Id) in strain BJ001. For strain AM1, a putative dihydrofolate reductase dfrB gene (META2_0242, 34 and 28% aa Id with DmrA and DfrA, respectively) is found in addition to the chromosomal gene; moreover, homologs to Mchl_1923 (META2_0462, 33% aa Id with the N-terminal domain), and CzcA2 (META2_1026, 85% aa Id with pCMU01 plasmid czcA2) are found.

<sup>b</sup>MaGe annotation (https://www.genoscope.cns.fr/agc/microscope).

<sup>c</sup>Precursors are uroporphyrinogen III and 5,6-dimethylbenzimidazole.

<sup>d</sup>n.d., not detected.

<sup>e</sup>Encode for homologs of different length: CobA (267 aa)/CysG (485 aa); CobC2 (519 aa)/CobC (338 aa); PurU (287 aa)/PurN (219 aa).

<sup>f</sup>In M. extorquens strains, H<sub>4</sub>F is synthesized either de novo or salvaged from 5,10-methylene-H<sub>4</sub>F, or 5- or 10-formyl-H<sub>4</sub>F [11,72,73].

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Table 3. Proteomic analysis of differentially expressed proteins in chloromethane- and methanol-grown cultures of *M. extorquens* CM4.

| Protein Description                                      | Protein Identifier | Gene | Protein parameters | Mass spectrometry identification data of different p ranges tested |
|----------------------------------------------------------|--------------------|------|--------------------|----------------------------------------------------------|
|                                                          |                    |      |                    | Score | Error (ppm) | Coverage (%) | Score | Error (ppm) | Coverage (%) | Score | Error (ppm) | Coverage (%) |
| Chloromethane utilization                               |                    |      |                    |       |             |              |       |             |              |       |             |              |
| CmuA, two-domain methyltransferase/corrinoid binding protein | Mchl_5697         | cmuA | CH₃Cl f             | 67.0  | 5.5        | 223          | 54    | 33          | 203          | 37    | 50          | 224          | 47    | 48          |
| CmuB, methylcobalamin H₄F methyltransferase (EC 2.1.1.86) | Mchl_5727         | cmuB | CH₃Cl f             | 33.3  | 5.1        | 203          | 21    | 57          | 142          | 27    | 47          | 130          | 33    | 49          |
| CobH₂, precorrin-8X methylmutase (EC 5.4.1.2)             | Mchl_5691         | cobH₂| CH₃Cl               | 22.0  | 5.1        | 203          | 28    | 85          | n.d. h | n.d. | n.d. | n.d. | n.d. | n.d. |
| MetF, 5,10-methylene-H₄F reductase (EC 1.5.1.20)          | Mchl_1881         | metF | CH₃Cl               | 34.1  | 6.6        | n.d.         | n.d. | n.d. | 170          | 63    | 47          | n.d.          | n.d. | n.d. |
| PaaE-like, oxidoreductase FAD/NAD(P)-binding domain protein | Mchl_5717         | paaE | CH₃Cl/+++           | 40.2  | 4.7        | 258          | 27    | 66          | 271          | 51    | 64          | 216          | 10   | 65          |
| PurU, formyl-H₄F hydrolase (EC 3.5.1.10)                  | Mchl_5699         | purU | CH₃Cl f             | 32.8  | 6.6        | n.d.         | n.d. | n.d. | 238          | 40    | 66          | 147          | 13   | 61          |
| Methylopathy                                              |                    |      |                    |       |             |              |       |             |              |       |             |              |
| Fae, formaldehyde-activating enzyme (EC 4.3.3.2)          | Mchl_2169         | fae  | – –                 | 18.1  | 5.7        | 108          | 47    | 42          | n.d.         | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Fch, methenyl-H₄F cyclohydrase (EC 3.5.4.9)               | Mchl_2134         | fch  | – – f               | 21.7  | 4.8        | n.d.         | n.d. | n.d. | 116          | 18    | 55          |
| Ftl, formate-H₄F ligase (EC 6.3.4.3)                      | Mchl_0447         | ftl  | +++                 | 59.5  | 6.8        | n.d.         | n.d. | n.d. | 348          | 46    | 58          | n.d.          | n.d. | n.d. |
| Hpr, hydroxypropane reductase, NAD(P)H-dependent (EC 1.1.1.29) | Mchl_2132         | hprA | – –                 | 34.2  | 5.2        | 152          | 46    | 39          | n.d.         | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MauB, methylamine dehydrogenase (EC 1.4.99.3) large subunit | Mchl_0565         | mauB | CH₃Cl/+++           | 44.7  | 7.2        | n.d.         | n.d. | n.d. | 270          | 49    | 66          | 168          | 11   | 49          |
| MtdA, bifunctional protein [NAD(P)H-dependent methylene-H₄MPT/methylene-H₄F dehydrogenase] (EC 1.3.1.⁄1.5.1) | Mchl_2133         | mtdA | – –                 | 29.7  | 7.0        | n.d.         | n.d. | n.d. | 121          | 43    | 34          |
| MtkA, malate thiokinase large subunit (EC 6.2.1.9)        | Mchl_2135         | mtkA | – –                 | 420   | 5.8        | n.d.         | n.d. | n.d. | 278          | 51    | 75          | n.d.          | n.d. | n.d. |
| MxhA, methanol dehydrogenase (EC 1.1.99.8) large subunit  | Mchl_4518         | mxhA | CH₃Cl/+++ f         | 68.4  | 5.9        | 129          | 16    | 19          | 364          | 63    | 54          | 82           | 8    | 20          |
| Spa, serine glyoxylate aminotransferase (EC 2.6.1.45)     | Mchl_2131         | spa  | – – f               | 43.2  | 6.9        | n.d.         | n.d. | n.d. | 283          | 24    | 78          |
| Central metabolism                                       |                    |      |                    |       |             |              |       |             |              |       |             |              |
| Acs, acetyl-CoA synthetase (EC 6.2.1.1)                   | Mchl_2785         | acs  | CH₃Cl/+++ f         | 72.2  | 5.6        | 413          | 38    | 53          | 374          | 53    | 55          | 231          | 16   | 41          |
| CbbA, fructose-bisphosphate aldolase (EC 4.1.2.13)        | Mchl_2046         | cbbA | CH₃Cl               | 38.6  | 5.5        | 92           | 37    | 28          | n.d.         | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| ClyK, cysteine synthase A and O-acetylseryl sulfhydroxylase A subunit (EC 2.5.1.47) | Mchl_0937         | clyK | CH₃Cl               | 34.5  | 5.9        | 186          | 37    | 64          | n.d.         | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Protein | Identifier | Gene | Protein parameters | Mass spectrometry identification data of different p/ranges tested |
|---------|------------|------|-------------------|---------------------------------------------------------------|
|         |            |      | **Ratio** | **M**<sub>r</sub> (kDa) | **p**<sub>f</sub> | **Score** | **Error** (ppm) | **Coverage** (%) | **Score** | **Error** (ppm) | **Coverage** (%) | **Score** | **Error** (ppm) | **Coverage** (%) |
|         |            |      | **CH<sub>3</sub>Cl/CH<sub>3</sub>OH** |               |               |               |               |               |               |               |               |               |               |               |
| EtfA, electron transfer flavoprotein subunit alpha | Mchl_1823 | etfA | 2 | 26.7 | 7.9 | n.d. | n.d. | n.d. | 26.7 | 7.9 | n.d. | n.d. | n.d. | n.d. |
| EtfB, electron transfer flavoprotein subunit beta | Mchl_1822 | etfB | 2 | 32.4 | 5.0 | n.d. | n.d. | n.d. | 32.4 | 5.0 | n.d. | n.d. | n.d. | n.d. |
| FumC, fumarase C (EC 4.2.1.2) | Mchl_2891 | fumC | CH<sub>3</sub>Cl | 49.8 | 5.6 | 129 | 81 | 51 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| GcvT, H<sub>4</sub>F-dependent aminomethyltransferase, glycine cleavage complex subunit (T protein) (EC 2.1.2.10) | Mchl_0814 | gcvT | CH<sub>3</sub>Cl | 40.3 | 6.0 | n.d. | n.d. | n.d. | 40.3 | 6.0 | n.d. | n.d. | n.d. | n.d. |
| GpX, fructose 1,6-bisphosphatase, class II (EC 3.1.3.11) | Mchl_2242 | gpxX | +++ | 34.6 | 5.4 | 146 | 44 | 41 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| HisA, phosphoribosylformiminono-5-aminoimidazole carboxamid ribotide isomerase | Mchl_2774 | hisA | CH<sub>3</sub>Cl | 26.7 | 5.4 | 183 | 27 | 42 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| HisD, bifunctional histidinal dehydrogenase and histidinol dehydrogenase (EC 1.1.1.23) | Mchl_2261 | hisD | CH<sub>3</sub>Cl | 45.4 | 5.0 | n.d. | n.d. | n.d. | 45.4 | 5.0 | n.d. | n.d. | n.d. | n.d. |
| His, homospermidine synthase (EC 2.5.1.44) | Mchl_5462 | his2 | CH<sub>3</sub>Cl | 53.2 | 5.3 | 246 | 37 | 40 | n.d. | n.d. | n.d. | 103 | 9 | 25 |
| Lpd, dihydrolipoamide dehydrogenase (EC 1.8.1.4) | Mchl_1930 | lpd | CH<sub>3</sub>Cl | 49.0 | 5.7 | 81 | 26 | 20 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MetK, 5-adenosylmethionine synthetase (EC 2.5.1.6) | Mchl_3629 | metK | | 41.8 | 5.4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 227 | 19 | 76 |
| NAD(P)H:quinone oxidoreductase (EC 1.6.5.2) | Mchl_4391 | qorB | CH<sub>3</sub>Cl | 38.2 | 7.8 | 254 | 31 | 63 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| NuoE, NADH-quinone oxidoreductase, chain F (EC 1.6.5.3) | Mchl_1210 | nuoE | | 44.6 | 4.8 | n.d. | n.d. | n.d. | 139 | 57 | 65 | n.d. | n.d. | n.d. |
| NuoF, NADH-quinone oxidoreductase, chain F (EC 1.6.5.3) | Mchl_1209 | nuoF | CH<sub>3</sub>Cl | 47.4 | 6.4 | n.d. | n.d. | n.d. | 217 | 30 | 49 | n.d. | n.d. | n.d. |
| PntA, NAD(P)+ transhydrogenase, subunit alpha part 1 (EC 1.6.1.2) | Mchl_2986 | pntA | CH<sub>3</sub>Cl | 39.6 | 5.6 | 177 | 35 | 45 | n.d. | n.d. | n.d. | 256 | 18 | 83 |
| ureidoglycolate lyase (EC 4.3.2.3) | Mchl_4377 | j | CH<sub>3</sub>Cl | 31.4 | 5.3 | 70 | 24 | 25 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| putative homoserine O-acetyltransferase (EC 2.3.1.31) | Mchl_4434 | j | | 42.1 | 5.9 | n.d. | n.d. | n.d. | 279 | 27 | 66 | n.d. | n.d. | n.d. |
| Adaptation to stress | | | | | | | | | | | | | | |
| KatA, catalase (hydroperoxidase II) (EC 1.1.1.6) | Mchl_3534 | katA | CH<sub>3</sub>Cl | 60.0 | 5.9 | 394 | 23 | 63 | 324 | 35 | 57 | 256 | 6 | 56 |
| MdoG, periplasmic glucan biosynthesis protein | Mchl_2321 | mdoG | CH<sub>3</sub>Cl | 58.7 | 5.6 | n.d. | n.d. | n.d. | 323 | 47 | 56 | n.d. | n.d. | n.d. |
| RfbC, dTDP-4-dehydrorhamnose 3,5-epimerase (EC 5.1.3.13) | | | | | | | | | | | | | | |
| SufS, selenocysteine lyase (EC 4.4.1.16) | Mchl_4348 | sufS | | 45.6 | 5.9 | 223 | 33 | 65 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
Table 3. Cont.

| Protein                                                                 | Identifier a Gene | Protein parameters | Mass spectrometry identification data b of different pI ranges tested |
|------------------------------------------------------------------------|-------------------|--------------------|-----------------------------------------------------------------------|
|                                                                       |                   | Ratio c CH3Cl/CH3OH | M_r (kDa) | pI | 4–7 | 3–10 | 3–10 NL d |
|                                                                       |                   | Score              | Error (ppm) | Coverage (%) | Score | Error (ppm) | Coverage (%) | Score | Error (ppm) | Coverage (%) | Score | Error (ppm) | Coverage (%) |
| SurE, 5’-nucleotidase (EC 3.1.3.5) Mchl_4603                          | surE             | CH3Cl              | 27.3 | 5.4 | 46 | 27 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| UspA-like, putative universal stress protein Mchl_1555                 | uspA             | ++                 | 29.3 | 6.1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 79 | 10 | 32 |     |
| putative manganese catalase (EC 1.11.1.6) Mchl_3002 j                 | CH3Cl            | 31.0 | 4.9 | 144 | 36 | 42 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Protein biosynthesis and modification                                  |                   |                    |        |     |     |     |     |     |     |     |     |     |     |     |
| AspS, aspartyl-tRNA synthetase (EC 6.1.1.12) Mchl_4374                 | aspS             | CH3Cl              | 67.2 | 5.5 | n.d. | n.d. | n.d. | 245 | 16 | 48 | n.d. | n.d. | n.d. | n.d. | n.d. |
| ClpP, ATP-dependent Clp protease, proteolytic subunit (EC 3.4.21.92)   | clpP             | --                 | 23.1 | 5.8 | n.d. | n.d. | n.d. | 181 | 40 | 71 | n.d. | n.d. | n.d. | n.d. | n.d. |
| EF-Ts, protein chain elongation factor Mchl_2348                       | tsf              | --                 | 32.3 | 5.5 | n.d. | n.d. | n.d. | 214 | 27 | 70 | n.d. | n.d. | n.d. | n.d. | n.d. |
| EF-Tu, protein chain elongation factor, GTP-binding factor Mchl_2438   | tuB              | --                 | 43.1 | 5.4 | n.d. | n.d. | n.d. | 86 | 32 | 28 | n.d. | n.d. | n.d. | n.d. | n.d. |
| Other functional classes                                               |                   |                    |        |     |     |     |     |     |     |     |     |     |     |     |     |
| ABC transporter, sulfate/thiosulfate transporter periplasmic protein Mchl_0592 | cysP             | CH3Cl              | 30.9 | 5.1 | n.d. | n.d. | n.d. | 254 | 31 | 63 | n.d. | n.d. | n.d. | n.d. | n.d. |
| ABC transporter, putative periplasmic substrate-binding protein Mchl_0388 | --               | CH3Cl              | 69.0 | 6.7 | 288 | 45 | 52 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| ABC transporter, putative substrate-binding protein, aliphatic sulphonates Mchl_0381 | --               | CH3Cl              | 34.3 | 8.1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 120 | 8 | 47 |     |
| conserved protein of unknown function Mchl_4437 j                      | --               | CH3Cl              | 16.1 | 5.5 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 234 | 21 | 26 |     |

aMaGe database (http://www.genoscope.cns.fr/agc/mage).
bProbability-based mouse score calculated using MASCOT software (Matrix Science, London, UK); error refers to mass accuracy; coverage refers to the percentage of the protein sequence covered by the matched peptides.

cSpots indicated as "CH3Cl" were only detected in the proteome of *M. extorquens* CM4 grown with chloromethane. Spots indicated as ''+'' were more abundant in chloromethane-grown cultures (or less abundant in methanol-grown cultures). Spots indicated as ''-'' were more abundant in methanol-grown cultures (i.e. less abundant in chloromethane-grown cultures). Factors of differential abundance were defined as follows: ++ (22^2 - 5) to 5-fold; +++ (22^2) more than 5-fold.

dNL, non linear pI range used in 2D-DIGE experiments.

eOnly found in strain CM4 (among the 8 *Methylobacterium* strains for which the complete genome sequence is known; [5,11]) and localized on plasmid pCMU01.

fMultiple spots detected.

gMass spectrometry used to discriminate from Mchl_1712 displaying 86% sequence identity at the protein level.

hMass spectrometry used to discriminate from Mchl_1712 displaying 86% sequence identity at the protein level.

iMass spectrometry used to discriminate from Mchl_2317 displaying 96% sequence identity at the protein level.

jNo assigned gene name.

kMass spectrometry data did not allow us to discriminate between two homologs with 99% sequence identity (Mchl_2669/Mchl_4004).

lTandem mass spectrometry identification.

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CM4 also contains plasmid-borne copies of 13 cob genes and genes coding for cobalt and preformed corrinoid transporters beyond the close chromosomal homologs of these genes shared by M. extorquens strains. These include the putative cobalt transporter CzcA-related RND transporter [27] (the plasmid-borne gene product Mchl_5715 displays 43% aa Id with the chromosome-encoded Mchl_1072; Table 2), and a homolog of the preformed corrinoid specific transporter Btu [28]. Unlike the plasmid-borne btu gene cluster, the chromosome-encoded btuFCD cluster lacks the btuB gene preceded by a cobalamin riboswitch [29] (Mchl_misc_RNA_1, Table 2), suggesting that expression of the plasmid-borne btu gene cluster is controlled by cobalamin in its coenzyme form (AdoCbl).

Experimental Identification of Gene Clusters Specific of the Chloromethane Response

Differential analyses of proteins extracted from chloromethane- and methanol-grown cultures of M. extorquens CM4 were performed using 2D-E and 2D-DIGE. Overall, 88 protein spots showing differences in abundance between the two compared conditions were detected, resulting in the identification of 49 proteins (Table 3; Fig. S1). In total, 33 proteins were specific of chloromethane-grown cultures, whereas sixteen proteins were more abundant in methanol-grown cultures.

Many of the identified proteins with differential abundance have known or suspected roles in chloromethane utilization and methylotrophy (Table 3). Many of these proteins allowed to define chloromethane-specific clusters encoding proteins more abundant during growth with chloromethane (Fig. 2, Clusters A–F), clusters responding both to chloromethane and methanol (Clusters G–H), and or to methanol only (Clusters I–J). The two-domain methyltransferase/corrinoid binding protein CmuA, the methylcobalamin:H4F methyltransferase CmuB, and the formyl-H4F hydrolase PurU shown to be essential for chloromethane metabolism in strain CM4, were identified in the chloromethane proteome only (Fig. S1) as expected [6,9,10]. Experimental evidence for chloromethane-enhanced expression of a protein involved in cobalamin biosynthesis (precorrin-8X methylmutase CobH2), and of a putative oxidoreductase with FAD/NAD(P)-binding domain encoded by a paaE-like gene often associated with cmu genes [2], was obtained here for the first time. Overall, only cluster A encoding proteins more abundant during growth with chloromethane (CmuA; CmuB; CobH2; PaaE-like; PurU; Hss2; Table 3) was localized on plasmid pCMU01.

Proteomic Identification of Stress-related Proteins

Upon dehalogenation, each mole of chloromethane yields one mole of hydrochloric acid with concomitant decrease in pH and increase in chloride concentration [7]. Chloromethane-associated
Figure 3. Gene redundancy in the biosynthesis of cofactors required for chloromethane utilization in *Methylobacterium extorquens* CM4. Cbi, cobinamide; Cbl, cobalamin; Ado, adenosyl; DMB, dimethylbenzimidazole; NaMN, nicotinate mononucleotide. AdoCbl and tetrahydrofolate are essential cofactors of the *cmu* pathway [6,9]. Transport and enzymatic reactions are shown with dotted and full arrows, respectively. Genes indicated in bold are located on the 380 kb plasmid pCMU01. Circled gene names encode proteins more abundant in chloromethane cultures. AdoCbl can be synthesized *de novo* by an aerobic biosynthesis pathway that incorporates cobalt (diamond), or obtained from a salvage pathway after internalization of preformed Cbi or Cbl. In prokaryotes, the cobalt needed for corrin ring synthesis may be incorporated into cells using the CorA transport system [69], the putative transmembrane proteins CbtA and CbtB [14], the Resistance-Nodulation-Division (RND)-type Co<sup>2+</sup>/Zn<sup>2+</sup>/Cd<sup>2+</sup> efflux system CzcA [27], or the Icu transporter [70]. The TonB-dependent Btu system imports preformed corrinoid compounds [28]. We hypothesize that BluB-related proteins link AdoCbl and H<sub>4</sub>F *de novo* synthesis.

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proteins with homologs characterized in *E. coli* for their role in osmoprotection were identified (Proteomic data, Table 3). Among these, protein MdG may be associated with metabolism of osmoregulated periplasmic glucans [30], the putative dTDP-4-dehydrohamonase 3,5-epimerase RhlC may be involved in the synthesis of surface polysaccharides [31], and the putative nucleotidase SurE may be associated with survival at high NaCl concentrations, as observed in *E. coli* [32], where the corresponding gene lies within a survival operon conserved in Gram-negative bacteria [33].

Production of reactive oxygen species also seems associated with chloromethane utilization. One representative of each class of catalases known to catalyze disproportionation of hydrogen peroxide (H$_2$O$_2$) [34] was more abundant in the chloromethane proteome. Mchl_3002 is a putative non-haem manganese-containing catalase, Mchl_3534 is a KatA-like protein, whose gene is found next to a putative H$_2$O$_2$ activator gene sharing 43% aa Id with *E. coli* OxyR. In *E. coli*, OxyR induces the Suf system (sulfur mobilization [Fe-S] cluster) to combat inactivation of the [Fe-S] lsc assembly system by H$_2$O$_2$ [35]. Moreover, *E. coli* mutants lacking the Suf machinery are hypersensitive to cobalt at high concentrations of 200 µM [36]. In this study, SuS$, a$ selenocysteine lyase homolog, was found more abundant in the chloromethane proteome. Similarly, the CysK cysteine synthase homolog more abundant in chloromethane cultures suggests the probable importance of reactivation systems to maintain chloromethane dehalogenase activity under aerobic conditions, as cysteine is involved in maintaining the catalytic activity and structure of many proteins with [Fe-S] clusters including ferredoxins [37].

Taken together, these data suggest that growth with chloromethane may elicit stress responses, and in particular an oxidative stress response.

**Discussion**

This work reports genomic and proteomic data demonstrating that *cmuA* and *cmuB* genes are plasmid-borne, and that plasmid pCMU01 drives the previously defined pathway for H$_4$F-mediated chloromethane dehalogenation [6]. Specifically, plasmid pCMU01 harbors cognate genes involved in chloromethane-associated H$_4$F metabolism not found in other *M. extorquens* genomes (folC2, folD, metF2 and purU; Table 1) [6,10].

H$_4$F metabolism is likely to be strongly modulated during growth on chloromethane since proteins linked to H$_4$F such as CmuA, CmuB, MetF and PurU were exclusively detected during growth with chloromethane activator (Table 3), whereas proteins associated with methanol oxidation with the metabolic intermediate formaldehyde and the C$_1$ carrier H$_4$MPT were more abundant during growth with methanol (proteins Fae, Fch and MtdA; Fig. 1).

Here, the interplay of chloromethane and other methylotrophic pathways was evidenced for the first time. Two components of the glycine cleavage complex involved in the conversion of H$_4$F and glycine to 3,10-methylene-H$_4$F [38], the key C$_1$ intermediate for entry in the serine cycle, were either more abundant with chloromethane or with methanol (GcvT and Lpd, respectively; Table 3). This suggests that enzymes implied in central metabolism such as the glycine cleavage complex might be involved in integrating contradictory signals during growth with C$_1$ compounds, to fine-tune metabolic conditions required for growth, and to even out variations in available carbon sources.

Our proteomic study also revealed that essential serine cycle enzymes (Sga, HprA and MtkA) were more abundant in methanol-grown cultures (Table 3). These enzymes are encoded by a chromosomal region (Fig. 2, cluster J), highly conserved in *Methylobacterium* [11]. Acetyl-CoA, glyoxylate and NADPH have been demonstrated to decrease binding of QscR, a key regulator of C$_1$ metabolism [39] to the $sgr$ promoter, thereby inhibiting transcription of the major operon of the serine cycle ($sgr$- *lpd-midA*-*fch*; [40]). The higher level of acetyl-CoA synthetase in chloromethane-grown cultures (Table 3) may explain the observed lower abundance of five enzymes encoded by cluster J.

**PaaE-like Oxidoreductase, PntAA, MetF and Acs are Proteins with Predicted Functions for Growth with Chloromethane**

Proteomic data provided first experimental evidence for the involvement of four previously undetected proteins, identified here as more abundant during growth with chloromethane, in chloromethane utilization.

The PaaE-like protein encoded by plasmid pCMU01 features a ferredoxin reductase-type FAD binding domain and a 2Fe-2S ferredoxin-type iron-sulfur binding domain. The PaaE-like protein is the only iron-sulfur enzyme more abundant in the chloromethane proteome. This PaaE-like oxidoreductase was suggested to be responsible for the observed methanethiol oxidase activity in the chloromethane-degrading strain, *Aminobacter lissarensis* CC495 [41,42]. It is also conceivable that the PaaE-like protein acts in the reactivation of the corrinoid cofactor from the inactive Co(I) to the Co(III) form (Fig. 1), as corrinoid-dependent methyltransferases are prone to inactivation by oxidation, and bacteria often require an efficient reactivation system to maintain such proteins in an active form [43,44]. The implication of PaaE in chloromethane utilization may be linked to the detection of genes associated with the oxidation stress response in chloromethane-grown *M. extorquens* CM4.

The transhydrogenase protein PntAA (Fig. 2 cluster D) couples the transfer of reducing equivalents between NAD(H) and NADP(H) to the translocation of protons across the membrane [45]. Previous transcriptomic and proteomic studies showed that PntAA was up-regulated in succinate- vs methanol-grown cultures of *M. extorquens* AM1 [46,47], indicating possible differences in energy and reducing equivalent production occurring in *M. extorquens* strains grown on different carbon sources. Here, the higher abundance of the PntAA complex may be the consequence of higher requirements for reducing equivalents coupled to proton extrusion during chloromethane assimilation.

The 5,10-methylene-H$_4$F reductase MetF identified in the chloromethane-proteome is the chromosome-encoded protein and not the plasmid-borne protein MetF2 previously shown to be essential for chloromethane utilization [6]. The protein product of *metF2* with a calculated pI of 9.5 is at the limit of the pH range studied in our experiments, which may explain why MetF2 was not detected here. In *E. coli*, MetF provides one-carbon precursors for methionine synthesis [48] and operates in the opposite direction of the chloromethane degradation pathway. If both MetF and MetF2 homologs share the same metabolites as substrates and products, regulatory processes in the expression of the corresponding genes arising from differences in availability of metabolites may explain the observed increased abundance of MetF with chloromethane. Further experiments are required to clarify the implications of MetF homologs in chloromethane metabolism.

Identification of the chromosome-encoded acetyl-CoA synthetase Acs as more abundant in the chloromethane proteome was initially surprising. This protein is predicted to catalyze ATP-dependent conversion of acetate to acetyl-CoA (78% aa Id with the characterized *Bradyrhizobium japonicum* Acs enzyme, [49]).
Acetyl-CoA is a key metabolite at the interface of C1 and multcarbon interconversions involving \( \text{H}_2 \text{MPT} \)-dependent \( \text{C}_1 \) substrate oxidation pathways, the serine cycle, and the ethylmalonyl-CoA pathway essential for growth with methanol [50]. The interconversion of central intermediates such as acetyl-CoA could thus be modulated upon growth with the \( \text{H}_2 \text{F} \)-dependent chloromethane oxidation pathway compared to growth with methanol, resulting in higher abundance of Acs in cells grown with chloromethane.

Plasmid-encoded BluB2: a Potential Link between \( \text{H}_2 \text{F} \) and AdoCbl Cocfactors of Chloromethane Utilization

BluB was demonstrated to catalyze two distinct enzymatic reactions of the AdoCbl biosynthetic pathway: i) conversion of cobinamide to Cbl as a cob(II)pyrrole a,c-diamide reductase (EC 1.16.8.1) [51]; ii) synthesis of the lower ligand of AdoCbl, dimethylbenzimidazole in Alphaproteobacteria Sinorhizobium meliloti [52] and Rhodospirillum rubrum [53]. Strain CM4 harbors two bluB homologs: bluB, conserved in all investigated \( M. \) extorquens chromosomes, and bluB2 located on plasmid pCMU01 (38% aa Id between BluB and BluB2; Table 2). BluB2 is highly similar to a characterized enzyme [54] that triggers the oxygen-dependent transformation of reduced flavin mononucleotide (FMNH2) in characterized enzyme [54] that triggers the oxygen-dependent transformation of reduced flavin mononucleotide (FMNH2) in dimethylbenzimidazole and D-erythrose 4-phosphate, a key precursor of chorismate and an intermediate in \( \text{H}_2 \text{F} \) biosynthesis (Fig. 3). Considering the strong level of sequence conservation with proteins of known function (62% aa Id with S. meliloti BluB), we speculate that gene bluB2 may be central for chloromethane assimilation by providing precursors for biosynthesis of essential cofactors of this metabolism.

Which Role for the Observed Vitamin B12-related Gene Redundancy in Chloromethane Metabolism?

Half of the genomes of sequenced prokaryotes that contain homologs of cobalt-utilizing enzymes also possess the AdoCbl biosynthetic pathway, while 90% of the remaining acquire external vitamin B12 via the BtuFCD transport system [55]. The genome of \( M. \) extorquens CM4 contains chromosomal- and plasmid-borne genes for both AdoCbl biosynthesis and corrinoid salvaging via the Btu transporter (Table 2). In addition, plasmid pCMU01 potentially encodes the capacity to remodel exogenous corrinoids, as suggested from the presence of gene cobO2 encoding the cob(II)pyrrole a,c-diamide adenosyltransferase enzyme [14] and of gene cobU2 encoding nicotine-nucleotide-dimethylbenzimidazole phosphoribosyltransferase (Fig. 3). We speculate that the presence of a variety of corrinoid salvaging pathways, possibly with different substrate affinities and expression profiles (e.g. in response to oxygen, cobalt, vitamin B12, or dimethylbenzimidazole availability), may supply \( M. \) extorquens CM4 with corrinoid coenzyme required for efficient chloromethane dehalogenation in different environments.

Gene duplications are known to correlate with adaptive interactions in prokaryotes by providing competitive advantages for adaptation on specific environmental conditions [56]. Here, cobA-encoded S-adenosyl-L-methionine-dependent uroporphyrinogen III methylase (SUMT) may catalyze the transformation of uroporphyrinogen III into precorrin-2 [57] (Fig. 3). SUMT activity may be provided by either CobA and CysG [58], but only CysG is conserved in \( M. \) extorquens sequenced strains. Plasmid-borne CobA protein, as a key branch point enzyme in the biosynthesis of modified tetrapyroles, may favor synthesis of AdoCbl over siroheme driven by chromosome-encoded CysG, control flux to AdoCbl precursor synthesis, and consequently facilitate growth of \( M. \) extorquens CM4 with chloromethane.

Plasmid pCMU01 encodes a full complement of genes for the corrinoid salvaging pathway, but contains only an incomplete set of genes for the oxygen-dependent AdoCbl de novo biosynthesis pathway. Genes cobB, cobF, cobG, cobN, cobS, cobT and cobV are missing compared to the canonical pathway characterized in \( P. \) denitricans [Fig. 3; Table 2; [14]]. However, other yet uncharacterized protein-encoding genes of plasmid pCMU01 may substitute for missing AdoCbl biosynthetic genes. For instance, as previously suggested in \( S. \) paucimobilis [59], chD located next to cmuBC encodes a typical oxygen-independent AdoCbl biosynthetic enzyme which, as the missing CobF, features a S-adenosylmethionine binding site. Protein ChID was reported as a cobalt-precorrin-6A synthase in the anaerobic AdoCbl biosynthesis pathway [60], but its potential role in the corresponding aerobic pathway remains to be investigated.

Surprisingly, out of more than 20 putative vitamin B12-related proteins encoded by plasmid pCMU01, only CobH2 was more abundant in the chloromethane proteome (Table 2). A possible explanation for this observation is that vitamin B12-related proteins are also required during growth with methanol, e.g. as cobalamin is an essential cofactor for MeaA (ethylmalonyl-CoA mutase) activity in the ethylmalonyl-CoA pathway for glyoxylate regeneration [61]. Whether redundant genes encoding vitamin B12-related protein in \( M. \) extorquens CM4 are expressed and functional remains to be evidenced.

Plasmid pCMU01 provides another example of bacterial clustering of genes encoding functional enzymes and cognate genes for cofactor biosynthesis (Fig. 2A). Such a genetic linkage was described for the insect symbiont \( H. \) obglinia which has one of the smallest genomes, but dedicates 7% of its proteome to cobalamin synthesis [62]. Similarly, Lactobacillus reuteri possesses a metabolic genomic island involved in 3-hydroxypropionaldehyde biosynthesis which associates cobalamin biosynthetic genes and genes of the anaerobic glycerol metabolism [63], most probably reflecting a cobalamin requirement for glycerol dehydratase activity [64], and the polyketide synthesizing bacterium \( S. \) thermophiles sp. DSM4137 has AdoCbl biosynthetic genes adjacent to a putative elaiophylin biosynthetic gene cluster that includes a gene encoding AdoCbl-dependent methylmalonyl-CoA mutase [59]. The genetic linkage of cmu and cob genes is likely to provide an evolutionary advantage for efficient bacterial growth with chloromethane by the cmu pathway.

Origin and Evolution of Plasmid pCMU01

The mosaic organization of plasmid pCMU01 suggests a history of carbon utilization-related gene acquisition for chloromethane and acetone. Multistep assembly of various genetic elements dedicated to chloromethane utilization in the cmu plasmid is supported by several observations: i) A 137 kb segment flanked by IS elements (ISMch8 and ISMch3 of IS5 and IS3 family, respectively) contains all hitherto identified chloromethane utilization genes; ii) A 33 kb segment between cmuBC and cmuA contains remnants of transposase genes (Mchl_5703 and Mchl_5711) and genes involved in corrinoid compound metabolism such as the cobU2 gene and the putative cobalt heavy metal efflux transporter Czc cluster (Fig. 2A); iii) Atypical genes encoding enzymes relevant to growth on chloromethane such as genes bluB2, cmuA, and cobA are located (Table 1; [65]); iv) The accCABR cluster encoding acetone carboxylase for carbon assimilation is flanked by mobile elements (Table 1 and data not shown); v) 10% of the predicted plasmid CDSs are mobile element-related sequences (Table 1); vi) Relics of genes driving plasmid replication
Based on these findings, strain DM4 was identified (cluster B in Fig. 2; cluster C in [16], methane-degrading strain CM4 and dichloromethane-degrading clusters responding specifically to dichloromethane in M. extorquens). When these five gene clusters responding specifically to chloromethane (Fig. 2), only the catabolic gene cluster essential for adaptation of M. extorquens DM4, only one chromosomal gene cluster common to chloromethane-degrading strain CM4 and dichloromethane-degrading strain DM4 was identified (cluster B in Fig. 2; cluster C in [16], suggested to be involved in cell structure). Based on these findings, the adaptive response to growth with chloromethane is clearly quite different from that for growth with dichloromethane, in line with the completely different pathways for metabolism of these two halogenated C1 compounds.

On a final note, it is striking that the organization of the cmu genes on plasmid pCMU01 of M. extorquens CM4 is different from that found in bacteria utilizing the cmu pathway known to date [2]. M. extorquens CM4 constitutes the first representative strain of the M. extorquens species for which a plasmid-encoded carbon utilization function has been clearly established, and its plasmid pCMU01 features the only known catabolic gene cluster for the metabolism of halogenated methanes that is not chromosome-borne [4,11,16,66]. The state of plasmid pCMU01 as an autonomous replicating episome may have enabled the efficient acquisition of relevant resources for growth with chloromethane, and the shaping of unique genetic features not observed in other genomes of chloromethane-degrading bacteria.

Supporting Information

Figure S1 2D-DIGE master gel image of total protein extracts from chloromethane- and methanol-grown M. extorquens CM4 labeled with Cy2 (internal standard). Highlighted spots (circles) displayed differential abundance between chloromethane and methanol conditions, and were identified by mass spectrometry. 1, CmuA; 2, CmuB; 3, PurU; 4, PaaE-like oxidoreductase; 5, Fch; 6, Sgc; 7, MtdA; 8, putative UspA-like protein; 9, KatA; 10, MetK; 11, Hsl; 12, Acs; 13, PntA; 14, putative endoribonuclease (Mchl_4437) (See Table 3 and Fig. 1 legend). (TIF)

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Author Contributions

Conceived and designed the experiments: FB SV. Performed the experiments: SR TN. Analyzed the data: SR FB. Contributed reagents/materials/analysis tools: TN SV FB. Wrote the paper: SR FB SV.
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