Methyl bromide (MeBr) is a fumigant used in the cultivation of soft fruits, vegetables, and flowers. Use of MeBr as a pesticide increases the yield and quality of crops without leaving behind toxic residues characteristic of more complex organo-pesticides. The majority of anthropogenic MeBr is produced in the United States, where 80% of it is used in fumigation treatments (23). The annual global flux of MeBr into the atmosphere from agricultural fumigation is approximately 16 to 48 Gg year\(^{-1}\) (13). Generally, bacterial soil sinks have been overlooked when global uptake rates have been estimated, simply because there is insufficient data available. The emissions of methyl chloride (MeCl) and MeBr into the atmosphere from natural and anthropogenic sources cause ozone depletion. MeBr is the main source in the atmosphere of bromide ions, which are 50 to 60 times more effective than chloride ions in converting ozone to oxygen. The reactions of chloride and bromide ions with stratospheric ozone have contributed to 20% of the Antarctic ozone hole (21).

Natural sources of MeBr are biomass burning, salt marshes, higher plants, phytoplankton, seaweed, fungi, and wetlands (9, 16, 26, 28, 41). In addition to its oxidation by tropospheric OH radicals (19), other biogeochemical sinks for MeBr include its dissolution from the atmosphere into the oceans, where it is destroyed by chemical and/or biological processes (10, 15, 44), and its consumption by bacteria in soils (12, 33, 34). Methyl halide-oxidizing bacteria have been isolated from soils (4, 12, 33, 34). Methyl bromide as a sole carbon and energy source. A single cmu gene cluster was identified in IMB-1 that contained six open reading frames: cmuC, cmuA, orf146, paaE, hutI, and partial metF. CmuA from IMB-1 has high sequence homology to the methyltransferase CmuA from *Methylbacterium chloromethanicum* and *Hyphomicrobium chloromethanicum* and contains a C-terminal corrinoid-binding motif and an N-terminal methyltransferase motif. However, cmuB, identified in *M. chloromethanicum* and *H. chloromethanicum*, was not detected in IMB-1.

Strain IMB-1, an aerobic methylotrophic member of the alpha subgroup of the *Proteobacteria*, can grow with methyl bromide as a sole carbon and energy source. A single cmu gene cluster was identified in IMB-1 that contained six open reading frames: cmuC, cmuA, orf146, paaE, hutI, and partial metF. CmuA from IMB-1 has high sequence homology to the methyltransferase CmuA from *Methylbacterium chloromethanicum* and *Hyphomicrobium chloromethanicum* and contains a C-terminal corrinoid-binding motif and an N-terminal methyltransferase motif. However, cmuB, identified in *M. chloromethanicum* and *H. chloromethanicum*, was not detected in IMB-1.

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Natural sources of MeBr are biomass burning, salt marshes, higher plants, phytoplankton, seaweed, fungi, and wetlands (9, 16, 26, 28, 41). In addition to its oxidation by tropospheric OH radicals (19), other biogeochemical sinks for MeBr include its dissolution from the atmosphere into the oceans, where it is destroyed by chemical and/or biological processes (10, 15, 44), and its consumption by bacteria in soils (12, 33, 34). Methyl halide-oxidizing bacteria have been isolated from soils (4, 7, 20), seawater (10), and forest leaf litter (6).

The facultative methylotroph strain IMB-1 was isolated from fumigated soil and is a member of the alpha subgroup of the *Proteobacteria*, within the genus *Aminobacter*. Phylogenetic analysis shows IMB-1 to cluster with the MeCl and MeBr utilizer, strain CC495, isolated from forest leaf litter (6). IMB-1 grows on methyl halides, methylated amines, and non-C\(_1\) compounds such as glucose and pyruvate (4), but no growth or oxidation was observed with methyl fluoride (MeF), methane, propyl iodide, dibromomethane, dichloromethane, or difluoromethane (4, 20, 32).

Bacteria that utilize MeCl as the sole source of carbon have also been isolated (7): *Hyptimicrobium chloromethanicum* CM2\(^T\) and *Methylbacterium chloromethanicum* CM4\(^T\) (18). Studies exploring the mechanism of MeCl metabolism in *M. chloromethanicum* CM4 have recently suggested a pathway for MeCl utilization (39, 40). It was shown that two polypeptides, of 67 and 35 kDa, were induced during growth on MeCl (40). MeCl-grown cells were also capable of dehalogenating MeBr and methyl iodide but not dichloromethane or chloroethane. This suggested that the enzyme(s) responsible for MeCl degradation was specific for monohalomethanes. No growth was observed with MeBr, presumably due to the greater toxicity of this compound. Transposon mutagenesis was used to create mutants that could not grow on MeCl. Genes containing the transposon insertion were then cloned and sequenced, and this information was used to develop biochemical assays. A pathway for MeCl degradation was then suggested that represents a novel catabolic pathway for aerobic methylotrophs (39).

The first step of this pathway involves CmuA, a 67-kDa polypeptide, which has a methyltransferase domain and a corrinoid-binding domain. The methyltransferase domain transfers the methyl group of MeCl to the Co atom of the enzyme-bound corrinoid group (methyltransferase I activity). A second polypeptide, CmuB, then transfers the methyl group onto tetrahydrofolate (H\(_4\)F), forming methyl-H\(_4\)F (methyltransferase II activity). This folate-linked methyl group is then progressively oxidized to formate and then CO\(_2\) to provide reducing equivalents for biosynthesis. Carbon assimilation presumably...
occurs at the level of methylene H$_4$F, which can feed directly into the serine cycle. Four genes, $cmuA$, $cmuB$, $cmuC$, and $purU$, were shown to be essential for growth on MeCl but not on other C$_1$ substrates. More recently, molecular studies of $H$. chloromethanicum CM2 have also identified the $cmu$ genes essential for growth on MeCl (17).

The aim of this study was to identify and sequence the IMB-1 genes involved in MeBr utilization. An insight into substrate binding and structure of the proteins from IMB-1 was achieved by aligning the IMB-1 sequence with sequences of previously characterized polypeptides from MeCl utilizers.

**Growth media.** IMB-1 was routinely cultured on 50 ml of ammonium nitrate mineral salts medium (42) at 30°C. Growth on MeBr (0.2%, vol/vol) was achieved in crimp-sealed 126-ml serum vials by pulsed additions of filter-sterilized gas in order to avoid toxicity associated with high initial concentrations of MeBr.

**Construction of DNA libraries from $H$. chloromethanicum CM2.** DNA was extracted from strain IMB-1 as previously described (22). Genomic libraries were constructed by cloning DNA from IMB-1 into pBluescript II K/S digested with the appropriate restriction enzyme. Fragments suitable for cloning were identified by probing Southern blots of IMB-1 DNA with radioactively labeled probes for $cmuA$ from $H$. chloromethanicum CM2 and $M$. chloromethanicum CM4 by methods described by Sambrook et al. (29). Southern blots were hybridized at 65°C and washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C (high stringency) or at room temperature (low stringency) for 1 h.

**DNA sequencing and analysis.** DNA sequencing was performed by cycle sequencing with the Dye Terminator Kit (PE Applied Biosystems, Warrington, United Kingdom), and the DNA was analyzed with a model 373A automated DNA sequencing system (PE Applied Biosystems). DNA sequences and derived amino acid sequences were analyzed with the Genetics Computer Group (GCG) Wisconsin Package, version 8.0.1-Unix. Similarity searches were performed with the gapped BLAST (Basic Local Alignment Search Tool) program (1) against public protein and gene databases (http://www.ncbi.nlm.nih.gov).

**Identification of methyltransferase genes in strain IMB-1.** Hybridization of $cmuA$ from $M$. chloromethanicum CM4 and $H$. chloromethanicum CM2 to IMB-1 genomic DNA indicated that IMB-1 contained genes with homology to $cmuA$ (data not shown). The Southern blot was also probed with $cmuB$ from $M$. chloromethanicum CM4 and $H$. chloromethanicum CM2. However, even at low stringency, no hybridization was seen. This suggested that a gene(s) with homology to $cmuB$ from $M$. chloromethanicum CM4 or $H$. chloromethanicum CM2 was not present in IMB-1.

**Cloning the methyltransferase gene $cmuA$ from strain IMB-1.** In order to clone $cmuA$ from IMB-1, a partial library was made with restriction enzyme ($Bam$HI)-digested fragments of between 5.5 and 6.5 kb, identified in probing experiments with $cmuA$ probes. These were ligated into pBluescript II K/S to create a library of 500 recombinant clones. Hybridization with a 750-bp $cmuA$ fragment from IMB-1, generated by PCR using primers designed from $H$. chloromethanicum and $M$. chloromethanicum $cmuA$ sequences, identified eight identical clones containing a 6.4-kb $Bam$HI fragment of IMB-1 chromosomal DNA (pIMB6) (Fig. 1).

**Sequence analysis of the IMB-1 methyltransferase gene cluster.** The sequence of the 6.4-kb $Bam$HI fragment of IMB-1 revealed a cluster of putative methyl halide utilization genes (Fig. 1). The open reading frames (ORFs) from IMB-1 were matched to proteins in the National Center for Biotechnology Information database. The guanine-plus-cytosine content of the cloned DNA from strain IMB-1 is 61.5 mol%, which is similar to that of the genus *Aminobacter* (62 to 64 mol%) (38), within which IMB-1 groups by 16S rRNA sequence analysis.
appears that IMB-1 has one methyltransferase gene cluster similar to the methyltransferase gene cluster of *H. chloromethanicum* CM2 (Fig. 1), whereas methyltransferase genes involved in MeCl metabolism are located on two clusters in *M. chloromethanicum* CM4 (39) (Fig. 1). In IMB-1, the arrangement of genes in the methyltransferase cluster is cmuA, cmuC, orf146, and paaE. This is similar to the arrangement of the corresponding genes in *H. chloromethanicum* CM2 (Fig. 1).

When IMB-1 is grown on MeBr, three specific polypeptides of 28 kDa, 32 kDa (of equal intensity), and 67 kDa (less intense) are induced (results not shown). The 67-kDa polypeptide is similar in size to the methyltransferase/corrinoid CmuA from *H. chloromethanicum* CM2 (AF281259), CmuA from *M. chloromethanicum* CM4 (AJ011316), and MtaA from *M. barkeri*. Similar residues are shaded in gray; identical residues are in black boxes. The putative zinc-binding motif identified in MtaA from *M. barkeri* is shown in boldface type below the alignment (14).

Alignments of proteins from IMB-1 with structurally characterized proteins allow for speculation on the structure, function, and potential binding site residues within the proteins. CmuC from IMB-1 has high homology to CmuC from *H. chloromethanicum* CM2 (40%) and *M. chloromethanicum* CM4 (36%) and also to MtaA, a methyltransferase protein from *Methanosarcina barkeri* (25%) (Fig. 2) (14, 39). MtaA is an isoenzyme that can transfer a methyl group from methyl cob(III)alamin, yielding methyl coenzyme M (11). MtaA binds zinc or cobalt to activate coenzyme M for methyl group attack from methyl cob(III)alamin (30, 31). Conserved histidine and cysteine residues for zinc or cobalt binding are also present in CmuC (Fig. 2). Therefore, it is possible that the putative methyltransferase CmuC also has methyltransferase II activity, like MtaA, and transfers the methyl group from CmuA methyl cob(III)alamin to H$_4$F, forming methyl-H$_4$F. A CmuB homolog was not detected in IMB-1, which suggests that CmuC operates as the CmuB methylcofactor:H$_4$F methyltransferase does in *M. chloromethanicum* CM4 or alternatively that the cmuB gene in IMB-1 has low homology to cmuB from *H. chloromethanicum* CM2 and *M. chloromethanicum* CM4 (35), and was therefore not detected by Southern blot hybridization.

CmuA from IMB-1 has a high degree of homology to CmuA from *M. chloromethanicum* (78%) and *H. chloromethanicum* (79%) and has two domains with the potential for methyl transfer and corrinoid binding. CmuA from IMB-1 is smaller (62 kDa) than CmuA from *H. chloromethanicum* CM2 (67 kDa) and *M. chloromethanicum* CM4 (67 kDa) and shows less homology in the corrinoid binding region. In the C-terminal (corrinoid binding) region of CmuA from IMB-1, the histidine residue in MetH corresponds to a glutamine (Q$^{296}$), as is the case in *H. chloromethanicum* and *M. chloromethanicum*, and the only other conserved residues are three glycines (G$^{297}$, G$^{298}$, G$^{330}$). This suggests that the binding of the corrin ring is weaker (18, 39).

Orf146 from IMB-1 shows 29% homology over 70 amino acids to the UvrA-ABC transporter from *Streptomyces coelicolor* (25) (Oliver et al., unpublished European Molecular Biology Laboratory accession number T35244). Orf146 has significant homology (50%) to the small ORF also between CmuA and a putative reductase from *H. chloromethanicum* CM2.

A large ORF (1,094 bp) encodes a homolog of PaaE, which has significant homology to a family of oxidoreductase proteins. The highest matches were PaaE from *Escherichia coli* (30% homology), which is involved in phenylacetic acid degradation (8), and VanB from *Pseudomonas* sp. strain HR199 (28%), which is a vanillate O-demethylase oxidoreductase (24). The putative reductases, PaaE homologs, from strain IMB-1 and *H. chloromethanicum* CM2 have significant homology to each other (50%). However, the C-terminal region of paaE from *H. chloromethanicum* CM2 has not been cloned. These putative reductases have significant homology to the class 1A dioxygenase family, in which the N terminus reveals a flavin mononucleotide/flavin adenine dinucleotide (FMN/FAD) binding site and there is an NAD binding domain in the center and a plant-type ferredoxin [2Fe-2S] domain in the C terminus (2, 3) (Fig. 3). The best-studied dioxygenase reductase is OphA1 from *Burkholderia cepacia* (previously known as *Pseudomonas cepacia*), which is involved in phthalate degradation. The structure of this reductase is known, and FMN/FAD, NAD, and [2Fe-2S] binding sites and other important conserved residues have been determined (5). In this reductase family, the conserved consensus for binding of the riboflavin-isoalloxazine ring is R-x-YSL and x-R-G-G-S (where x is either G or S). The arginine in x-R-G-G-S is required for the binding of the FMN phosphate group, specifically binding FMN rather than FAD. In IMB-1 and *H. chloromethanicum* CM2, the residues within the conserved sequence (R-x-YSL) are present. However, the arginine residue in the x-R-G-G-S motif, specifically required for FMN binding, is not present, suggesting that the cofactor FAD is required. An NADH or NADPH binding
motif (G-x-G-x-x-P) is present in IMB-1 and *H. chloromethanicum* CM2 with related reductase sequences. The putative reductase (Orf165) from IMB-1 and the partial putative reductase (Orf240) from *H. chloromethanicum* CM2 have been aligned with OphA1 (phthalate dioxygenase reductase) from *B. cepacia* (AF095748), VanB from *Pseudomonas* sp. strain HR199 (Y11521), TsaB (toluene sulfonate methyl-monooxygenase reductase) from *Comamonas testosteroni* (U32622), and PaaE from *E. coli* (X97452). Similar residues are shaded in gray; identical residues are in black boxes. The FMN/FAD, NAD, and [2Fe-2S] ferredoxin conserved binding sites are indicated underneath the sequence, and other conserved residues are indicated by bullets (●) (3, 5).

FIG. 3. Alignment of the putative reductase (PaaE) proteins from IMB-1 and *H. chloromethanicum* CM2 with related reductase sequences. The putative reductase (Orf165) from IMB-1 and the partial putative reductase (Orf240) from *H. chloromethanicum* CM2 have been aligned with OphA1 (phthalate dioxygenase reductase) from *B. cepacia* (AF095748), VanB from *Pseudomonas* sp. strain HR199 (Y11521), TsaB (toluene sulfonate methyl-monooxygenase reductase) from *Comamonas testosteroni* (U32622), and PaaE from *E. coli* (X97452). Similar residues are shaded in gray; identical residues are in black boxes. The FMN/FAD, NAD, and [2Fe-2S] ferredoxin conserved binding sites are indicated underneath the sequence, and other conserved residues are indicated by bullets (●) (3, 5).

The HutI homolog homology to an imidazolonepropionase from *Sinorhizobium meliloti* that belongs to the HutI family of proteins involved in histidine degradation (43). The N-terminal domain of the HutI homolog from IMB-1 has 35% homology and 55% similarity to Orf165 (encoded by a partially cloned gene) from *M. chloromethanicum* CM4. However, a link between this protein and the dehalogenation of methyl halides is not known, and it may be that the imidazole ring found in the nucleotide loop of the cobalamin structure needs to be degraded during the dehalogenation reaction.

Previously it has been proposed that methyl-H$_4$F, produced from MeCl by methyltransferase activity, is then reduced by the metylene-H$_4$F reductase (MetF), identified in *M. chloromethanicum* CM4 (39). A putative MetF has also been identified in strain IMB-1, which has homology with MetF from *M. chloromethanicum* CM4 (33%) and MetF from *Saccharomyces cerevisiae* (23%) (36). Methylene-H$_4$F would be a key intermediate in the degradation of halomethanes by *M. chloromethanicum* CM4 and IMB-1, as this substrate can be either oxidized to formate or converted by serine transhydroxymethylase and assimilated into cell biomass via the serine cycle.

**Nucleotide sequence accession number.** The sequence of the cmu gene cluster from strain IMB-1 has been deposited in GenBank (accession number AF281260).

We acknowledge the financial support provided by the Natural Environment Research Council (GR9/2192) and for studentships to C. Woodall and K. Warner and INTAS grant 94–3122. We thank Don Kelly (University of Warwick) for useful comments on the manuscript.

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