Dichloromethane metabolism and C₁ utilization genes in *Methylobacterium* strains

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The ability of methylotrophic α -proteobacteria to grow with dichloromethane (DCM) as source of carbon and energy has long been thought to depend solely on a single cytoplasmic enzyme, DCM dehalogenase, which converts DCM to formaldehyde, a central intermediate of methylotrophic growth. The gene *dcmA* encoding DCM dehalogenase of *Methylobacterium dichloromethanicum* DM4 was expressed from a plasmid in closely related *Methylobacterium* strains lacking this enzyme. The ability to grow with DCM could be conferred upon *Methylobacterium chloromethanicum* CM4, a chloromethane degrader, but not upon *Methylobacterium extorquens* AM1. In addition, growth of strain AM1 with methanol was impaired in the presence of DCM. The possibility that single-carbon (C₁) utilization pathways in dehalogenating *Methylobacterium* strains differed from those discovered in strain AM1 was addressed. Homologues of tetrahydrofolate-linked and tetrahydromethanopterin-linked C₁ utilization genes of strain AM1 were detected in both strain DM4 and strain CM4, and cloning and sequencing of several of these genes from strain DM4 revealed very high sequence identity (96.5–99.7%) to the corresponding genes of strain AM1. The expression of transcriptional *xylE* fusions of selected genes of the tetrahydrofolate- and tetrahydromethanopterin-linked pathways from strain DM4 was investigated. The data obtained suggest that the expression levels of some C₁ utilization genes in *M. dichloromethanicum* DM4 grown with DCM may differ from those observed during growth with methanol.

Keywords: methylotrophy, dehalogenase, chlorinated methanes

INTRODUCTION

Dichloromethane (DCM) is a solvent produced and used industrially in large quantities which easily escapes into the environment because of its volatile and water-soluble character (McKay *et al.*, 1993; van Agteren *et al.*, 1998). This is a source of concern because of the genotoxic effects associated with DCM in bacteria as well as in mammals (Kayser *et al.*, 2000; Landi, 2000; Kayser & Vuilleumier, 2001; Vuilleumier, 2002).

Methylobacterium dichloromethanicum DM4 is able to use DCM as the sole source of carbon and energy (Galli & Leisinger, 1988; Doronina *et al.*, 2000; Vuilleumier, 2002). The first step in the degradation of DCM is catalysed by DCM dehalogenase, a bacterial represen-

tative of the glutathione *S*-transferase family of enzymes (Vuilleumier & Pagni, 2002), which converts DCM to hydrochloric acid and formaldehyde, a central intermediate of methylotrophic growth (Fig. 1). The ability of *Methylobacterium* strains to use DCM for growth was originally thought to only require possession of this enzyme. However, emerging evidence now suggests that additional genes and proteins may also be necessary for DCM metabolism (Vuilleumier, 2002). In particular, the genotoxic effects associated with DCM-dehalogenase-dependent conversion of DCM (Kayser & Vuilleumier, 2001) indicate that an efficient DNA repair system is required. Indeed, DNA polymerase I, an enzyme well known to participate in several DNA repair mechanisms, was shown to be essential for growth with DCM in strain DM4 (Kayser *et al.*, 2000).

Two parallel pterin-linked formaldehyde utilization pathways were recently shown to be essential for energy generation and carbon assimilation in *Methylobacterium extorquens* AM1 growing with methanol (Chis-

Abbreviations: DCM, dichloromethane; H₄folate, tetrahydrofolate; H₄MPT, tetrahydromethanopterin; MeOH, methanol.

The GenBank accession numbers for the sequences determined in this work are AJ421476 and AJ421477.

Fig. 1. Current model of C₁ metabolism in *Methylobacterium*. Formaldehyde is produced in the periplasm by a pyrroloquinoline-quinone-dependent methanol dehydrogenase (MxaFI) or in the cytosol by DCM dehalogenase (DcmA). As proposed by Chistoserdova *et al.* (1998), cytoplasmic formaldehyde reacts with either H₄folate or H₄MPT, and the C₁ unit is further oxidized to CO₂ or incorporated into cell biomass via the serine cycle. Fae, formaldehyde-activating enzyme (Vorholt *et al.*, 2000); MtdA, methylene H₄folate/H₄MPT dehydrogenase (Vorholt *et al.*, 1998; Hagemeier *et al.*, 2001); MtdB, methylene H₄MPT dehydrogenase (Hagemeier *et al.*, 2000); FchA, methenyl-H₄folate cyclohydrolase; MchA, methenyl-H₄MPT cyclohydrolase (Pomper *et al.*, 1999); fts, formyl-H₄folate synthase; Ftr, formylmethanofuran H₄MPT formyltransferase encoded by *ftsA*, which is in complex with the gene products of *orf123* (Pomper & Vorholt, 2001); *fdh*, formate dehydrogenase. Dotted arrows denote steps that are as yet poorly characterized.

toserdova *et al.*, 1998) (Fig. 1). One of these pathways is tetrahydrofolate (H₄folate) dependent and has so far only been described for strain AM1 (Vorholt *et al.*, 1998; Pomper *et al.*, 1999). The second pathway is tetrahydromethanopterin (H₄MPT) dependent and appears to be present in many methylotrophic bacteria (Vorholt *et al.*, 1999).

The presence and the involvement of these two pathways for growth with halogenated methanes in strains closely related to *M. extorquens* AM1 such as *M. dichloromethanicum* strain DM4 and the chloromethane-degrading strain *M. chloromethanicum* CM4 (Vannelli *et al.*, 1999) (97–98% identity in 16S rDNA sequences) remained to be investigated. Notably, the pathway for chloromethane mineralization in *M. chloromethanicum* CM4 (Vannelli *et al.*, 1999; McDonald *et al.*, 2001) is unlikely to involve formaldehyde as an intermediate. Two proteins of strain CM4, CmuA and CmuB, were shown to catalyse the dehalogenation of chloromethane by a vitamin-B12-mediated methyl group transfer to H₄folate (Studer *et al.*, 2001). Several lines of evidence also suggest that C₁ units in strain CM4 are then further oxidized to formate by a series of enzymes specific to the chloromethane-degrading strain (A. Studer, C. McAnulla & S. Vuilleumier, unpublished results).

In this study, we first investigated whether the expression of the DCM dehalogenase from a plasmid was sufficient to support growth of *M. extorquens* AM1 or of *M. chloromethanicum* CM4 with DCM as the sole carbon

source. We then addressed the question whether the recently described genes for C₁ utilization of strain AM1 were also present in the dehalogenating strains DM4 and CM4, and if so, whether such genes were expressed during growth with DCM.

METHODS

Materials. Restriction and DNA-modifying enzymes used for cloning were from Fermentas. Oligonucleotides were purchased from Microsynth (Balgach, Switzerland). All other chemicals were analytical grade or better and were purchased from Fluka except where noted.

Bacterial strains and growth conditions. *E. coli* strains DH5α (Ausubel *et al.*, 2001), XL-1 Blue (Bullock *et al.*, 1987) and S17-1 (Simon *et al.*, 1983) were grown on LB medium (Ausubel *et al.*, 2001) at 37 °C with ampicillin (100 mg l⁻¹) or tetracycline (25 mg l⁻¹) as required. *Methylobacterium* strains were grown at 30 °C in liquid minimal medium (MM) with methanol (MeOH; 40 mM), DCM (10 mM) or succinate (10 mM) as carbon sources as described elsewhere (Kayser *et al.*, 2000). Bacterial growth in liquid culture was determined by monitoring the optical density of the cultures at 600 nm.

Matings. Plasmids were mobilized into *M. dichloromethanicum* DM4-2cr (Gälli & Leisinger, 1988), *M. extorquens* AM1 (DSM 6343) and *M. chloromethanicum* CM4 (McDonald *et al.*, 2001) by biparental mating using *E. coli* S17-1 (Simon *et al.*, 1983) as the donor strain. Cultures of donor (OD₆₀₀ 0.5) and recipient (OD₆₀₀ 1.0) were mixed in a 1:2 ratio, spotted on nutrient agar medium (Difco) and incubated overnight at 30 °C. Bacteria were resuspended in 0.9% sodium chloride, washed, diluted and plated on selective MM agar

plates containing the required antibiotics and MeOH (40 mM) or succinate (10 mM) as the carbon source.

Hybridization analysis. Preparation of total DNA, DNA digestion and hybridization analysis were performed by standard procedures (Ausubel *et al.*, 2001). Specific DNA probes for *mchA*, *fae* and *mtaA* genes were generated by PCR using digoxigenin (DIG)-labelling PCR mix (Roche Diagnostics), specific primers for the required genes and chromosomal DNA of *M. extorquens* AM1 as a template. Dimethylsulfoxide was added to all PCR reactions at a final concentration of 6% (v/v). For *mchA*, a 443 bp DIG-labelled DNA fragment of *M. extorquens* AM1 was generated using primers TACAAGGA-GCTCGGCTATCG and GAAAAGATCTCGGCGAAGG (Vorholt *et al.*, 1999) (all primer sequences are given in the 5' to 3' direction). For *fae* detection, a 248 bp fragment of strain AM1 was generated using degenerate primers GGCGAGGC-SCTSGTCGGSGAYGG and GCGACGCCATGYTSGSCS-GGSCC (Vorholt *et al.*, 2000). A 850 bp *mtaA* probe was generated from plasmid pCH2 (Hagemeyer *et al.*, 2000) with standard primers T7 (TAATACGACTCACTATAGGG) and T7term (GCTAGTTATTGCTCAGCGG). All probes were purified by agarose gel electrophoresis using the QIAquick gel extraction kit (Qiagen). After hybridization at 68 °C overnight, the membrane (HybondN, Amersham) was washed under stringent conditions and detection was performed according to the manufacturer's instructions. The membrane was stripped with 0.2 M NaOH, 0.1% SDS at 37 °C for 10 min and stored at 4 °C for subsequent use.

Construction of DCM dehalogenase expression plasmids. Broad-host-range plasmids were constructed for expression of the DCM dehalogenase (*dcmA*) of *M. dichloromethanicum* DM4. Plasmid pME8220 was constructed by cloning the 1.5 kb *HindIII*-*PstI* fragment containing *dcmA* without its negative transcriptional regulator *dcmR* (La Roche & Leisinger, 1991), from plasmid pME1540 (Schmid-Appert, 1996), into *HindIII*/*PstI*-digested and dephosphorylated broad-host-range cloning vector pCM62 (Marx & Lidstrom, 2001). Plasmid pME8221 was obtained by cloning the 2 kb *Bam*HI fragment containing *dcmA* and its negative transcriptional regulator *dcmR* from plasmid pME1518 (La Roche & Leisinger, 1990) into *Bam*HI digested and dephosphorylated pCM62.

Cloning of C₁ utilization genes from *M. dichloromethanicum* DM4. The 5.8 kb *Bam*HI-*Eco*RV fragment hybridizing with the *mchA* probe was cloned into *Bam*HI/*Eco*RV-digested and dephosphorylated pBluescript II KS(+) (Stratagene), yielding plasmid pME8167. For sequencing purposes, the internal 2.8 kb *Nco*I fragment from pME8167 was cloned into *Nco*I-digested and dephosphorylated pUC29 (Benes *et al.*, 1993), yielding plasmid pME8168. Plasmid pME8169 was obtained by *Nco*I digestion and religation of plasmid pME8167. The 4.8 kb *Nco*I fragment hybridizing with a *mchA* probe was cloned into *Nco*I-digested and dephosphorylated pUC29, yielding plasmid pME8067. Subclones were generated by religation of *Bam*HI- or *Sac*II-digested pME8067, yielding pME8068 and pME8069, respectively. The 1.6 kb *Bam*HI insert fragment of plasmid pME8067 was cloned into *Bam*HI-digested and dephosphorylated pBluescript II KS(+), yielding plasmid pME8070. The 4.8 kb *Nco*I fragment hybridizing with the *mtaA* probe was cloned into *Nco*I-digested and dephosphorylated pUC29, resulting in plasmid pME8161. Subclones pME8163 and pME8164 were constructed by religation of a 3.6 kb *Not*I and a 2.6 kb *Eco*RI fragment of pME8161, respectively. Plasmids pME8165 and pME8166 were generated by subcloning a 2 kb *Eco*RI and a 1 kb *Not*I fragment, from pME8161, respectively, into pBluescript.

Sequence analysis. Clones and subclones were sequenced using standard primers T7, T3 (ATTAACCCCTCACTAAAG-G) and M13rev (CAGGAAACAGCTATGACC) and by primer walking using Dye-Terminator chemistry and an ABI 377 Automated Sequencer or an ABI 310 Genetic Analyser (Perkin-Elmer). Sequences were analysed with the Genetics Computer Group sequence analysis package (version 10). Similarity searches against public protein and gene databases were performed using gapped BLAST (Altschul *et al.*, 1997). The sequences obtained and analysed here were submitted to the EMBL database under accession numbers AJ421476 and AJ421477.

Construction of plasmids with transcriptional *xylE* fusions.

The broad-host-range promoter probe vector pCM130 (Marx & Lidstrom, 2001) was used to construct plasmid derivatives with DNA sequences from *M. dichloromethanicum* DM4 found upstream of the genes *hprA* (292 nt, plasmid pME8230), *mtaA* (157 nt, pME8231), *sgaA* (746 nt, pME8232), *mchA* (799 nt, pME8233) and *dcmA* (689 nt, pME8226) fused to a promoterless *xylE* gene. DNA sequences upstream of genes of interest were amplified from suitable plasmids. PCR products digested with either *Eco*RI (*hprA*, *mtaA*) or *Bam*HI (*sgaA* and *mchA*), using *Bam*HI sites present in the amplified products, see Fig. 3) and *Hind*III were cloned into vector pCM130 digested with the same enzymes. This afforded gene fusions displaying the gene-upstream DNA sequence of interest fused 6 nt upstream of the RBS sequence (GGAGG) and 18 nt (in frame) upstream of the start ATG codon of the promoterless *xylE* reporter gene. The sequences of the primers were (5' to 3' direction, introduced restriction site in bold): GACCCCGAA-TTCGGGTGTTGCCGCCGCTC and TCGTGTAAGCT-TCAAACCCCTGCCGCCCGT for *hprA*, CGCAGGAA-TTCGTCGAGGCGTAAGAAACGCA and GCTTCTAAG-CTTTGATTCTCTGGCCGGTTG for *mtaA*, GATGCA-GATCGGACAGGAAG and TCGTTGAAGCTTTGAGAA-TTTCCTCCAATTAA for *sgaA*, CGCAAGATGCTGGAG-GCG and TGTGGAAGCTTTGATTGTTCTCCGTTTC-C for *mchA*, TTTCTTAAGCTTTCCGTTTCTTCCTCGC-AAGG and GATTCGAAGCTTCGTTATCCTCCCTTAC-TGTG for *dcmA*, respectively. The resulting plasmids, as well as pCM137, a pCM130 derivative with the DNA region upstream of *fae* from *M. extorquens* AM1 fused to the *xylE* gene (C. J. Marx & M. E. Lidstrom, personal communication), were transferred from *E. coli* S17-1 into *M. dichloromethanicum* DM4 by conjugation.

Preparation of cell extracts. Bacterial cultures (50 ml) were harvested at early stationary phase by centrifugation at 12000 g for 15 min at 4 °C, resuspended in 3 ml ice-cold extraction buffer (50 mM Tris/HCl pH 7.5, 1 mM GSH, 2 mM DTT, 0.5 mM EDTA, 25%, v/v, glycerol) and cells disrupted by three passages through a French pressure cell (55 MPa). After centrifugation to remove cell debris (35000 g, 45 min, 4 °C), protein concentration in cell-free extracts was determined using a commercial Bradford reagent (Bio-Rad) and bovine serum albumin (Sigma) as a standard.

Enzyme assays. Specific DCM dehalogenase activity was measured in triplicate (standard error < 15% of the mean) in cell extracts (2–20 µg protein) using a previously described coupled enzyme assay with formaldehyde dehydrogenase (Vuilleumier & Leisinger, 1996). The reaction was performed at 30 °C in gas-tight spectrophotometric cuvettes in 100 mM sodium/potassium phosphate pH 8. For measurement of catechol dioxygenase activity, cultures were grown in 60 ml minimal medium containing 25 mg tetracycline l⁻¹ in 300 ml flasks with gas-tight screw-caps (Supelco) at 30 °C under shaking (160 r.p.m.) with 10 mM succinate, 10 mM DCM or

40 mM MeOH as the carbon source. Samples (1 ml late-exponential culture; OD₆₀₀ 0.4–0.8) were centrifuged, resuspended in 1 ml cold catechol-2,3-dioxygenase (Xyle) assay buffer (50 mM potassium phosphate pH 7.5, 10% acetone) and changes in absorbance upon addition of 10 µl 0.1 M 1,2-dihydroxybenzene were recorded at 375 nm and 25 °C. Specific activity of Xyle was expressed as nmol product formed per min per 10⁸ cells, using an absorption coefficient (ϵ_{375}) of 10⁴ M⁻¹ cm⁻¹ for the reaction product (Rotmel *et al.*, 1991) and a value of 10⁸ cells per ml culture of OD₆₀₀ 1 (unpublished observations).

RESULTS

Expression of DCM dehalogenase in *Methylobacterium* strains

Our current investigations on DCM metabolism aim at defining which metabolic features are important for growth of methylotrophic bacteria with DCM. We were therefore interested in whether the expression of the DCM dehalogenase from *M. dichloromethanicum* DM4 in highly related *Methylobacterium* strains would allow them to grow with DCM. The *dcmA* gene encoding the DCM dehalogenase of strain DM4 was expressed from the recently described shuttle vector pCM62 (Marx & Lidstrom, 2001) in *M. dichloromethanicum* DM4-2cr, *M. chloromethanicum* CM4 and *M. extorquens* AM1, which are all unable to grow with DCM (Fig. 2a). Strain DM4-2cr is a mutant of strain DM4 with a large (>20 kb) chromosomal deletion including the *dcmA* gene. This mutant is known to grow with DCM when provided with a plasmid carrying the *dcmA* gene (Galli & Leisinger, 1988) and was thus used here as a control. Two different constructs were used for DCM dehalogenase expression. Plasmid pME8221 (Fig. 2c) allows expression of the structural gene *dcmA* under the control of its upstream negative transcriptional regulator *dcmR* (La Roche & Leisinger, 1991), while plasmid pME8220, containing *dcmA* with a truncated upstream DNA region lacking the *dcmR* regulator gene, leads to constitutive expression of DCM dehalogenase (Fig. 2b).

Sustained growth with DCM as the sole carbon source was observed in transconjugants of *M. dichloromethanicum* DM4-2cr and *M. chloromethanicum* CM4, but not of *M. extorquens* AM1 (Fig. 2b, c). Maximal growth rates ($\mu_{\max}$) in transconjugants were slightly reduced compared to those of wild-type strains, possibly as a consequence of the presence of antibiotics in the medium. However, all transconjugant strains expressed active DCM dehalogenase as determined by measurements of specific activity in cell-free extracts. DCM dehalogenase activity *in vivo* was confirmed by release of chloride ions into the growth medium in the presence of DCM (data not shown).

As expected from previous work (La Roche & Leisinger, 1991), expression of DCM dehalogenase was regulated by DCM and *dcmR*. DCM dehalogenase expression in transconjugants with plasmid pME8221 carrying *dcmA* and its transcriptional regulator *dcmR* was dependent on the presence of DCM (Fig. 2c). A tenfold increase in DCM dehalogenase activity was observed in transcon-

Fig. 2. Growth and DCM dehalogenase expression in different *Methylobacterium* strains. Maximal growth rates ($\mu_{\max}$) and specific DCM dehalogenase activity was measured in cultures of strains unable to grow with DCM (a); in transconjugants of these strains constitutively expressing the DCM dehalogenase gene *dcmA* from plasmid pME8220 lacking the *dcmR* regulator gene (b); and in transconjugants containing plasmid pME8221 with *dcmA* under the control of *dcmR* (c). Cultures were grown with MeOH (white), a mixture of MeOH and DCM (hatched) or with DCM (black). Growth rates represent the mean of at least two experiments differing by no more than 10%.

jugants from all strains when DCM was added to MeOH-grown cultures. Transconjugants growing with DCM alone showed an even higher induction of DCM dehalogenase expression. In pME8220 transconjugants which lacked *dcmR*, in contrast, DCM dehalogenase was constitutively expressed in MeOH- and MeOH/DCM-grown cultures (Fig. 2b). This was expected from previous investigations (La Roche & Leisinger, 1991; Schmid-Appert, 1996) and confirmed that the *dcmR* gene is involved in the repression of DCM dehalogenase expression in the absence of DCM. Strikingly, however, the constitutively expressed DCM dehalogenase activity of the DM4-2cr(pME8220) transconjugant observed with MeOH was increased when DCM was used as the

Table 1. Size of DNA fragments containing *mtdA*, *mchA* and *fae* homologues in *Methylobacterium* strains as deduced from Southern blot analysis

Gene	Fragment	Size (kb)		
		AM1	DM4	CM4
<i>fae</i>	<i>Pst</i> I	3.6	> 10	> 10
	<i>Sal</i> I	5.3	> 10	5.3
	<i>Nco</i> I	1.3	3	5.6
	<i>Sma</i> I	7.0	> 10	> 10
<i>mtdA</i>	<i>Pst</i> I	9	9	> 10
	<i>Sal</i> I	5.7/1.0	5.7/1.0	5.7
	<i>Nco</i> I	5.5	4.6	5.5
	<i>Sma</i> I	6.2	6.2	6.2
<i>mchA</i>	<i>Pst</i> I	8.2	8.2	> 10
	<i>Sal</i> I	7.5	> 10	9.0
	<i>Nco</i> I	3.0	3.0	5.6
	<i>Sma</i> I	6.9	> 10	> 10

sole source of carbon and energy (Fig. 2d). This suggests the existence in strain DM4 of an additional level of regulational control to that described previously (La Roche & Leisinger, 1991) which involves the DCM-dependent induction of *dcmA*.

Surprisingly, expression of the DCM dehalogenase in strain AM1 did not support growth of this strain with DCM (Fig. 2). On the contrary, the mere presence of DCM in MeOH-grown cultures of strain AM1 transconjugants was detrimental to growth (Fig. 2). Such cultures grew very slowly ($\mu_{\max} < 0.01$) and to only a low final optical density ($OD_{600} \sim 0.1$). Slower and poorer growth was only observed in *M. extorquens* AM1 expressing active DCM dehalogenase, since the maximal growth rate of the wild-type *M. extorquens* AM1 lacking a *dcmA*-containing plasmid was only slightly reduced in the presence of DCM (Fig. 2a).

Taken together, these data suggested that the ability of *Methylobacterium* strains to utilize DCM as the sole source of carbon and energy was not solely dependent on the reaction catalysed by DCM dehalogenase. The possibility that pathways of C₁ utilization may differ in *Methylobacterium* strains other than AM1 was therefore investigated by probing the dehalogenating strains DM4 and CM4 for the presence of the genes encoding enzymes of the C₁ utilization pathways recently characterized in *M. extorquens* AM1 (Chistoserdova *et al.*, 1998).

Cloning and sequence analysis of formaldehyde utilization genes in *M. dichloromethanicum* DM4

Hybridization analysis demonstrated that genes of H₄folate-linked and H₄MPT-linked C₁ utilization pathways were also present in *M. dichloromethanicum* DM4 and *M. chloromethanicum* CM4, although the sizes of the hybridizing fragments often differed (Table 1 and

data not shown). The 4.6 kb *Nco*I fragment of *M. dichloromethanicum* DM4 containing the methylene H₄folate/H₄MPT dehydrogenase gene *mtdA* was cloned and sequenced (Fig. 3a). As in *M. extorquens* AM1, *mtdA* from strain DM4 is clustered together with the genes for serine glyoxylate aminotransferase (*sgaA*) and hydroxypyruvate reductase (*hprA*), encoding enzymes of the serine cycle (Chistoserdova & Lidstrom, 1994). The genes encoding methenyl-H₄MPT cyclohydrolase (*mchA*) and formaldehyde-activating enzyme (*fae*) were also cloned from strain DM4. Again, these genes are organized as in strain AM1 (Chistoserdova *et al.*, 1998) (Fig. 3b). Analysis of the cloned genes revealed a high sequence identity of 96.5% to 99.7% to the corresponding genes of *M. extorquens* AM1 (Fig. 3). Interestingly, similar levels of sequence conservation were also observed in putative intergenic regions (98.9%), suggesting that these genes represent a major metabolic module in a dehalogenating *Methylobacterium* strain as well.

Expression of C₁ utilization genes in *M. dichloromethanicum* DM4 during growth with different carbon sources

The expression of selected genes cloned from strain DM4 that were likely to be associated with C₁ utilization was then investigated. Plasmid-borne transcriptional *xylE* fusions were constructed using the promoter-probe vector pCM130 recently described for analysis of gene expression in *M. extorquens* AM1 (Marx & Lidstrom, 2001). Constructs containing DNA sequences located upstream of the genes *sgaA*, *hprA*, *mtdA* and *mchA* of *M. dichloromethanicum* DM4, PCR-amplified from appropriate plasmids (Fig. 3), were cloned into pCM130 and the resulting derivatives conjugated into the wild-type DM4 strain. Catechol dioxygenase activity encoded by the reporter gene *xylE* was determined in these transconjugants during growth of strain DM4 with different carbon sources (Fig. 4). Transconjugants with the vector pCM130 itself, with plasmid pCM137 containing a transcriptional fusion of the *fae* promoter sequence from *M. extorquens* AM1 (C. J. Marx, personal communication), and with pME8226, a pCM130 derivative containing the promoter sequence of the DCM dehalogenase gene *dcmA* as a positive control, were also investigated. The DNA sequence upstream of the *fae* gene in strain DM4 was found to be identical to that of strain AM1, so that plasmid pCM137 actually represented a homologous probe for monitoring the expressions of the *fae* gene in the DM4 background.

The DNA sequence upstream of the *mchA* gene (Fig. 4), as well as those upstream of the *hprA* and *mtdA* genes (data not shown), did not give rise to XylE activity above the background observed with the promoterless control pCM130. These findings were not unexpected in the case of the *mtdA*'-*xylE* construct, which was previously suggested to be cotranscribed with the upstream *hprA* gene. However, the sequence upstream of the *hprA* gene was previously suggested to contain a promoter for *hprA* (Chistoserdova & Lidstrom, 1994).

Fig. 3. Gene cluster analysis of *C*₁ utilization genes in *M. dichloromethanicum* DM4. The percentage of sequence identity to the corresponding genes in *M. extorquens* AM1 is given at protein and DNA levels. Genes known to be essential for growth of *M. extorquens* AM1 with MeOH (Chistoserdova & Lidstrom, 1992, 1994; Chistoserdova *et al.*, 1998; Vorholt *et al.*, 2000) are marked in black. Restriction sites unique to strain DM4 are marked in bold. (a) Methylene-*H*₄folate/*H*₄MPT dehydrogenase (*mtdA*); serine glyoxylate aminotransferase (*sgaA*); hydroxypyruvate reductase (*hprA*). (b) Methylene-*H*₄MPT dehydrogenase (*mtdB*); methenyl-*H*₄MPT cyclohydrolase (*mchA*); formaldehyde-activating enzyme (*fae*).

Fig. 4. Expression of *C*₁ utilization genes in *M. dichloromethanicum* DM4 during growth with different carbon sources: Xyle activity in *M. dichloromethanicum* DM4 transconjugants carrying plasmids with transcriptional *xylE* fusions grown with succinate (S, white), MeOH (M, hatched) and DCM (D, black). The data represent the mean of three independent experiments (standard errors are shown by error bars).

As for the sequence upstream of *mchA*, it contains a putative gene *orfY* (Fig. 3) whose disruption in strain AM1 did not lead to a growth phenotype with *C*₁ compounds as growth substrates (Chistoserdova *et al.*, 1998). The *mchA'*-*xylE* construct was therefore used to investigate whether this region contained promoter activity for *mchA* expression.

In contrast, the sequence upstream of the serine glyoxylate aminotransferase gene *sgaA* caused significantly higher expression of the reporter gene during growth with MeOH than with DCM (Fig. 4). Further, DNA sequences upstream of *fae* and *dcmA* genes led to similar *xylE* expression with MeOH and DCM as the *C*₁ growth substrate, but also with succinate (Fig. 4). Constitutive expression of the *fae'*-*xylE* fusion was somewhat unexpected considering the key role of the *H*₄MPT-dependent formaldehyde-activating enzyme Fae for

processing formaldehyde during growth of strain AM1 with MeOH (Vorholt *et al.*, 2000). However, the observed constitutive expression of *xylE* under the control of the *dcmA* promoter was in keeping with previous observations with plasmid-borne transcriptional *lacZ* fusions of sequences upstream of *dcmA* (Schmid-Appert, 1996) that also lacked the *dcmR* regulator gene (La Roche & Leisinger, 1991).

DISCUSSION

DCM-dehalogenase-mediated dehalogenation of DCM imposes several severe constraints on bacterial metabolism. Formaldehyde, the product of dehalogenation, certainly constitutes a toxic burden to the methylotrophic cell (Anthony, 1982). However, DNA alkylation by S-chloromethylglutathione, the probable intermediate in the dehalogenation of DCM, was shown to have more deleterious effects than formaldehyde in strain DM4 (Kayser *et al.*, 2000; Kayser & Vuilleumier, 2001; Vuilleumier, 2002).

The results reported here indicate that expression of active DCM dehalogenase from a plasmid supports growth with DCM of *M. dichloromethanicum* DM4-2cr and *M. chloromethanicum* CM4 but not of *M. extorquens* AM1. For reasons still unknown, therefore, *M. extorquens* AM1 appears ill-suited to cope with the metabolic consequences of DCM metabolism. The existence of pathways for energy generation and *C*₁ assimilation from DCM in dehalogenating *Methylobacterium* strains that differed from those of strain AM1 represented one possibility that could help to rationalize this finding. The work presented here, however, clearly shows that many of the genes implicated in both pathways of *C*₁ oxidation and assimilation recently discovered in *M. extorquens* AM1 (Chistoserdova *et al.*, 1998; Vorholt *et al.*, 1999) are conserved with very high sequence identity in strain DM4 (Fig. 3). It of course remains to be excluded that dehalogenating strains employ additional pathways for formaldehyde detoxification, such as the linear glutathione-dependent formaldehyde dehydrogenase/formyl-glutathione hydrolase pathway found in *Paracoccus denitrificans* for

example (van Spanning *et al.*, 2000; Goenrich *et al.*, 2002). In our view, however, the most attractive hypothesis for the lack of growth of strain AM1 expressing DCM dehalogenase with DCM would be a lesser resistance of this strain to intracellular acid production compared to dehalogenating *Methylobacterium* strains. This idea is currently under investigation. Alternatively, strain AM1 could express some proteins absent from dehalogenating strains which could lead to deleterious toxic effects in the presence of DCM. Such proteins would not be unheard of: for example, some methane monooxygenases from methanotrophic bacteria were shown to accept DCM as a substrate, leading to the formation of reactive and therefore toxic products (see e.g. Murrell, 1994). Bacteria of the *Methylobacterium* genus, however, are unable to grow with methane and lack methane monooxygenase. Yet another possibility worth investigating in the future would be that strain AM1 is optimized for coping with formaldehyde production in the periplasm by methanol dehydrogenase (Fassel *et al.*, 1992) rather than in the cytoplasm by DCM dehalogenase (Leisinger *et al.*, 1994).

Whatever the reason behind the lack of growth of strain AM1 with DCM, the *xylE* gene fusions investigated so far (Fig. 4) could not pinpoint C₁ utilization genes displaying higher expression levels during growth with DCM compared to MeOH-driven growth. Indeed, the reverse was observed in the case of *sgaA*, which encodes an enzyme of the serine cycle of carbon assimilation. Of course, deducing expression patterns from experiments with plasmid-borne gene fusions can be a risky undertaking. In the absence of any information on transcriptional initiation, however, the precise placement of the gene fusion of the start codon of the gene of interest directly upstream of the RBS of the *xylE* reporter, as performed here, ensured that spurious effects arising from altered stability of the reporter mRNA (Haas, 2001) would be kept to a minimum. The titration of regulator proteins by high-copy plasmids containing regulatory DNA sequences is another potential problem associated with plasmid-based reporters of gene expression. In the present case, however, DNA hybridization data suggested that the promoter-probe plasmid used, pCM130, was reproducibly and stably present in four to six copies in the cell (unpublished observations). Unfortunately, satisfactory gene integration systems remain to be developed for strain DM4. Indeed, preliminary experiments aiming at the insertional inactivation of genes in strain DM4 by using non-replicating plasmids derived from pAYC61 (Chistoserdov *et al.*, 1994), as commonly performed in the background of *M. extorquens* AM1 (Chistoserdova *et al.*, 1998; Vorholt *et al.*, 2000), have been unsuccessful.

In conclusion, the present study confirms previous indications (Kayser *et al.*, 2000) that factors other than DCM dehalogenase are required for growth of *Methylobacterium* strains with DCM. The analysis of the expression of genes involved in C₁ utilization now emerges as an attractive aspect of research on this subject.

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