Plasmid Analysis and Cloning of the Dichloromethane-utilization Genes of *Methylobacterium* sp. DM4

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The dichloromethane (DCM)-utilizing facultative methylotroph *Methylobacterium* sp. DM4 was shown to contain three plasmids with approximate sizes of 120 kb, 40 kb and 8 kb. Curing experiments suggested that the DCM-utilization character was correlated with the possession of an intact 120 kb plasmid. The DCM-utilization genes were cloned on the broad-host-range vector pVK100. Plasmid pME1510, a recombinant plasmid carrying a 21 kb *Hind*III fragment complemented DCM-utilization-negative derivatives of *Methylobacterium* sp. DM4 and conferred the DCM-utilization-positive phenotype to a number of Gram-negative methylotrophic bacteria. In Southern hybridization experiments with pME1510 as a probe, chromosomal DNA from *Methylobacterium* sp. DM4 gave definite signals while purified plasmid DNA did not. Plasmid pME1510 did not hybridize with total DNA from a cured DCM-non-utilizing derivative of *Methylobacterium* sp. DM4. It is concluded that the DCM-utilization genes are located on the chromosome or on a megaplasmid. Curing procedures thus led to the formation of a chromosomal or megaplasmid deletion larger than 21 kb and covering the DCM-utilization genes or to the loss of an undetected megaplasmid.

INTRODUCTION

Soil bacteria isolated after enrichment for growth on dichloromethane (DCM) as the sole carbon and energy source have been shown to comprise facultative methylotrophs of the genera *Pseudomonas* (Brunner *et al.*, 1980; Gälli & Leisinger, 1985; LaPat-Polasko *et al.*, 1984) and *Hyphomicrobium* (Stucki *et al.*, 1981). Some of the DCM-utilizing *Pseudomonas* isolates are pink-pigmented facultatively methylotrophic bacteria which, according to a recent taxonomic study (Green & Bousfield, 1983), should be placed in the genus *Methylobacterium*. This is the case for *Methylobacterium* sp. strain DM4 which is the subject of the present communication and formerly was named *Pseudomonas* sp. DM4 (Gälli & Leisinger, 1985; Kohler-Staub *et al.*, 1986). The ability to grow on DCM depends on the possession of DCM dehalogenase. This enzyme, a hexamer with a subunit M_r of approximately 33000, requires reduced glutathione as a cofactor and converts one molecule of DCM into one molecule of formaldehyde and two molecules of hydrochloric acid. It is highly inducible by its substrate and constitutes about 16% of the total soluble protein of induced cells (Kohler-Staub & Leisinger, 1985).

The purified DCM dehalogenases of two *Hyphomicrobium* strains, one *Pseudomonas* sp., and of *Methylobacterium* sp. DM4 have recently been compared. They were immunologically similar, exhibited the same subunit M_r and were identical with respect to the 15 *N*-terminal amino acids (Kohler-Staub *et al.*, 1986). These observations suggested that the DCM dehalogenase structural gene and a putative regulatory gene were horizontally distributed among facultatively methylotrophic bacteria. In the present communication we describe the

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Abbreviations: DCM, dichloromethane; Tc, tetracycline; Km, kanamycin; Sm, streptomycin.

Table 1. *Bacterial strains and plasmids*

Genetic symbols for *E. coli* strains are standard. Dcm⁺, DCM-utilization-positive phenotype; PPFM, pink-pigmented, facultatively methylotrophic bacterium.

Strain or plasmid	Genotype or relevant properties	Source or reference
<i>E. coli</i>		
HB101	F ⁻ <i>pro leu hsdR hsdM recA supE rpsL</i> (Sm ^r)	Boyer & Roulland-Russoix (1969)
LE392	F ⁻ <i>met hsdR hsdM supE supF</i>	Maniatis <i>et al.</i> (1982)
BHB2688	N205 <i>recA</i> (λ imm ⁴³⁴ <i>cIts b2 red Eam Sam</i> /λ)	Hohn (1973)
BHB2690	N205 <i>recA</i> (λ imm ⁴³⁴ <i>cIts b2 red Dam Sam</i> /λ)	
<i>Protaminobacter ruber</i> (ATCC 8457)	Dcm ⁻ , PPFM	Green & Bousfield (1982)
<i>Pseudomonas extorquens</i> (DSM 1337)	Dcm ⁻ , PPFM	
<i>Pseudomonas</i> sp. M27 (DSM 1339)	Dcm ⁻ , PPFM	
<i>Methylobacterium organophilum</i> XX (ATCC 27886)	Dcm ⁻ , PPFM	
<i>Methylobacterium</i> sp. DM4	Dcm ⁺ , PPFM (pME1511, pME1512, pME1513)	Gälli & Leisinger (1985)
DM4-2c	Dcm ⁻ (pME1512, pME1513)	Cured derivative of DM4, this study
DM4-2cr	Dcm ⁻ Sm ^r (pME1512, pME1513)	Sm ^r derivative of DM4-2c, this study
DM4-161	Dcm ⁺ (pME1511, pME1512)	Spontaneous derivative of DM4, this study
DM4-161c	Dcm ⁻ (pME1514)	Cured derivative of DM4-161, this study
AM1(ATCC1478)	Dcm ⁻ , PPFM	Green & Bousfield (1982), Peel & Quayle (1961)
AM1r	Dcm ⁻ Rif ^r	Rif ^r derivative of AM1, this study
Plasmids		
pVK100	Km ^r Tc ^r , mobilizable cloning vector derived from RK2 containing the cohesive ends of the phage λ	Knauf & Nester (1982)
pRK2013	Km ^r , self-transmissible derivative of RK2 containing ColE1 replicon and transfer functions necessary to mobilize pVK100 and its derivatives	Figurski & Helinski (1979)
pME1510	Tc ^r Dcm ⁺ recombinant plasmid of pVK100 containing a 21 kb <i>Hind</i> III fragment from DM4	This study
pME1511	120 kb cryptic plasmid of DM4	
pME1512	40 kb cryptic plasmid of DM4	
pME1513	8 kb cryptic plasmid of DM4	
pME1514	50 kb cryptic plasmid of DM4-161c	
pME1515	Km ^r recombinant plasmid of pVK100 containing a 7 kb <i>Sal</i> I fragment of pME1511	

cloning of the DCM-utilization genes of *Methylobacterium* sp. DM4 and their expression in DCM-non-utilizing facultative methylotrophs. We also present evidence that these genes are not located on any of the three plasmids we have detected in *Methylobacterium* sp. DM4.

METHODS

Bacterial strains, plasmids and culture conditions. The bacteria and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C on LB medium (Maniatis *et al.*, 1982).

Methylobacterium sp. DM4 was grown at 30 °C in batch cultures on a rotary shaker with methanol or DCM as the carbon and energy source. The minimal medium used and the cultivation on the toxic and volatile substrate DCM have been described elsewhere (Kohler-Staub *et al.*, 1986). Methylamine agar contained (l⁻¹) 0.68 g

$\text{CH}_3\text{NH}_2 \cdot \text{HCl}$, 1.36 g KH_2PO_4 , 1.78 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.2 g $(\text{NH}_4)_2\text{SO}_4$, 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 15 g agar. DCM indicator agar was used for selection and detection of DCM degrading recombinant clones; it contained (l^{-1}) 0.54 g KH_2PO_4 , 0.2 g $(\text{NH}_4)_2\text{SO}_4$, 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g bromthymol blue and 15 g agar at pH 7.0. After sterilization of the solid media described above, trace elements were added as described by Stucki *et al.* (1981). The DCM indicator plates were incubated in a desiccator whose atmosphere contained DCM. The optimum DCM concentration in the air of the closed container was obtained by including a reagent tube with 0.1 ml DCM per litre of air volume. Bacterial degradation of DCM was accompanied by the release of hydrochloric acid which led to a zone of colour change from dark-green to yellow around degradative colonies.

Antibiotic concentrations (ml^{-1}) were 25 μg for tetracycline (Tc), 50 μg for kanamycin sulphate (Km), and 200 μg for streptomycin sulphate (Sm) and rifamycin (Rif).

Isolation of antibiotic-resistant mutants of *Methylobacterium* sp. DM4. Nutrient agar plates containing 20 μg Rif or Sm ml^{-1} were inoculated with 10^9 bacteria from liquid cultures. After 6 to 10 d incubation, resistant colonies were picked and streaked on nutrient agar plates with increased antibiotic concentrations. This procedure was repeated several times and led to strains resistant to 200 μg Rif ml^{-1} or 500 μg Sm ml^{-1} , corresponding to a 10-fold and 100-fold increase, respectively, in the MIC.

Curing experiments. *Methylobacterium* sp. DM4 was grown for 20 generations in liquid minimal medium with either 10 mM-methanol or 10 mM-succinate (sodium salt) as the sole carbon and energy source. To obtain Dcm⁻ mutants the cells were either incubated at the maximum growth temperature of 32 °C or at 30 °C in the presence of 1 μg mitomycin C ml^{-1} . Samples of the grown cultures were plated on methylamine agar. After 6 to 8 d incubation, single colonies were picked and transferred to methylamine plates and DCM indicator plates to give the proportion of Dcm⁻ derivatives.

Isolation and manipulation of DNA. Preparative amounts of plasmid DNA were isolated from *Escherichia coli* HB101 by the method of Itoh *et al.* (1984). For analytical purposes, cosmids were isolated from *E. coli* HB101 by the alkaline lysis method described by Maniatis *et al.* (1982). Total cellular DNA from *Pseudomonas aeruginosa* PAO1 and *Methylobacterium* sp. strains was purified by the method of Chesney *et al.* (1979).

Methylobacterium sp. plasmid DNA was isolated essentially by the method of Kado & Liu (1981). Methanol-grown cells (1 l) were harvested by centrifugation (5200 g, 20 min) and washed twice with saline (0.85%, NaCl). The pellet was resuspended in 20 ml E-buffer (40 mM-Tris/HCl, 2 mM-EDTA, pH 7.9) and 40 ml lysis buffer (50 mM-Tris, 10 mM-EDTA, 1%, w/v, SDS, pH 13.0) were added. The cell suspension was incubated at 65 °C for 10 min and quickly chilled on ice. Cold 2 M-Tris/HCl buffer (pH 7.0; 12 ml) and cold 5 M-NaCl (18 ml) were added, and the lysate was incubated on ice for 3 h. Cell wall debris and the bulk of chromosomal DNA were separated by centrifugation (16800 g, 20 min) and the pink, clear supernatant was extracted twice with phenol/chloroform (100 g phenol and 0.1 g hydroxyquinoline dissolved in 100 ml chloroform saturated with 100 mM-Tris/HCl, pH 8.0) and once with chloroform. DNA was precipitated by adding 10% (v/v) polyethylene glycol 6000 and 3% (w/v) NaCl. Plasmid DNA was further purified by centrifugation to equilibrium in CsCl-ethidium bromide gradients (Maniatis *et al.*, 1982).

Restriction endonucleases (Boehringer Mannheim and Pharmacia), T4 DNA ligase (Boehringer Mannheim) and bacterial alkaline phosphatase (Boehringer Mannheim) were used according to the manufacturer's recommendations. Digested total cellular and plasmid DNA were separated on horizontal 0.5–0.7% agarose gel with TAE buffer (Maniatis *et al.*, 1982) at 2 V cm^{-1} for 16 h and stained in ethidium bromide (5 mg l^{-1}). Gels were exposed on the top of a UV transilluminator ($\lambda = 254 \text{ nm}$) for 20 min and then soaked twice for 15 min in 0.25 M-HCl. DNA transfer to Hybond-N nylon membranes (Amersham) was done according to the method of Smith & Summers (1980). Filter hybridization was done according to the manufacturer's directions for Hybond-N, using ^{32}P -labelled, nick-translated DNA (Rigby *et al.*, 1977).

Cosmid cloning. Bacteriophage λ packaging extracts were prepared and *in vitro* packaging was performed according to Hohn (1973) as modified by Maniatis *et al.* (1982). The extracts were tested with λcI857 Sam7 DNA and *E. coli* LE392 as an indicator strain, and packaging efficiencies of $> 10^7$ p.f.u. ($\mu\text{g DNA}$)⁻¹ were achieved. Purified *Methylobacterium* sp. DM4 total cellular DNA (which was mixed with plasmid DNA from the same strain) was subjected to partial digestion with *Hind*III [300 $\mu\text{g DNA ml}^{-1}$, 0.10 U *Hind*III ($\mu\text{g DNA}$)⁻¹; 20 min at 37 °C according to Maniatis *et al.* (1982). DNA was size fractionated on a 0.5% agarose gel (0.5 V cm^{-1} , 15 h), and DNA fragments with a length of 15 to 28 kb were cut out of the gel with a sterilized razor blade. The fragments were electrophoretically separated from agarose in an electrophoretic chamber (Biotrap; Schleicher and Schuell) and precipitated with ethanol. The cosmid vector pVK100 was cut with *Hind*III and treated with alkaline phosphatase. Insert and vector DNA were ligated with T4 DNA ligase (0.4 mg DNA ml^{-1} , 200 U T4 ligase ml^{-1} ; 16 h at 14 °C; 3:1 ratio of vector to insert DNA). The ligated DNA was packaged and the phage particles adsorbed to *E. coli* HB101 as described by Maniatis *et al.* (1982). Typically, 10^4 Km^s Tc^r colonies were obtained from 1 μg ligated DNA. The HB101 clones were grown in freezing medium (Schleif & Wensink, 1981) containing Sm and Tc and stored at -70 °C.

Mobilization of cosmids and identification of recombinant clones. The ability of cosmids to complement Dcm⁻ mutants was tested by mobilizing the cosmids with the helper plasmid pRK2013 from *E. coli* HB101 into *Methylobacterium* sp. DM4-2cr. Multiples of 48 recombinant *E. coli* HB101 donors containing pVK100 with

inserts, arranged in a grid pattern on a master plate, were replica-plated onto a lawn of *E. coli* HB101(pRK2013) grown on nutrient agar. Matings were incubated at 37 °C for 9 h and then replica-plated onto nutrient agar plates containing Km, Tc and Sm. After incubation at 37 °C overnight these plates were patched onto a lawn of *Methylobacterium* sp. DM4-2cr grown on nutrient agar. After incubation at 30 °C for 48 h the *Methylobacterium* plates were replica-plated onto methylamine agar + Tc + Sm and onto DCM indicator agar + Tc + Sm. Clones growing on both methylamine and DCM were analysed for plasmid content and for DCM dehalogenase production by the antibody assay.

Rabbit antiserum against purified DCM dehalogenase of *Hyphomicrobium* sp. strain DM2 (Kohler-Staub *et al.*, 1986) was used to detect clones of *Methylobacterium* sp. DM4 which produce DCM dehalogenase. Clones grown on agar plates were patched onto nitrocellulose filters (Schleicher and Schuell) and lysed by the method of Konheiser *et al.* (1984). Antiserum against the DCM dehalogenase (diluted 10³-fold) was used as a primary antibody and 10³-fold diluted peroxidase-labelled anti-rabbit goat IgG (Sigma) as the secondary antibody. The binding of the secondary antibody was made visible by developing the filters with 4-chloro-1-naphthol and H₂O₂ (Konheiser *et al.*, 1984).

DCM dehalogenase assay. Cell-free extracts were prepared as previously described (Kohler-Staub *et al.*, 1986) and protein concentrations were measured by the method of Bradford (1976). DCM dehalogenase activity in the extracts was determined by the enzyme assay described by Stucki *et al.*, 1981).

RESULTS

Plasmid patterns and the Dcm⁻ phenotype

Methylobacterium sp. DM4 is one of ten DCM-utilizing facultative methylotrophs we have analysed for plasmid content. These strains included *Hyphomicrobium* sp. DM2, in which we were unable to detect plasmid DNA, eight *Pseudomonas* sp., each containing a plasmid of approximately 120 kb, and *Methylobacterium* sp. DM4, which exhibited the most complex plasmid pattern. Strain DM4 harboured plasmids pME1511, pME1512 and pME1513 with approximate sizes of 120 kb, 40 kb and 8 kb, respectively (Fig. 1). Since the plasmid pattern and the DCM-utilization character of this bacterium were readily changed by a curing treatment, this organism was used to study whether or not the DCM-utilization genes are plasmid-encoded. Two representative DCM-degrading *Pseudomonas* strains, which were also examined, did not yield Dcm⁻ mutants upon a variety of curing procedures.

Fig. 1. Plasmid content of DCM-degrading positive (Dcm⁺) and negative (Dcm⁻) derivatives of *Methylobacterium* sp. strain DM4 after growth under curing conditions. Crude plasmid preparations were isolated as described in the text. Lanes: 1, DM4 (Dcm⁺); 2, DM4-2cr (Dcm⁻); 3, DM4 (Dcm⁺); 4, DM4-161c (Dcm⁻).

Fig. 2. DCM dehalogenase in different Dcm^- mutants of *Methylobacterium* sp. strain DM4 as detected by the antibody assay. Cells were grown on methylamine agar plates, and the DCM dehalogenase protein was assayed as described in the text. Patches: 1-7, Dcm^- mutants derived from temperature-curing on methanol (7, DM4-161c); 8-17, Dcm^- mutants derived from temperature-curing on succinate (9, DM4-2cr); 18 and 19, Dcm^- mutants derived from mitomycin-C-curing on succinate; 20, *Methylobacterium* sp. strain DM4 (Dcm^+).

Table 2. Loss of DCM-degrading activity by *Methylobacterium* sp. strain DM4 under plasmid-curing conditions

Conditions		Percentage of Dcm^- mutants after growth for 20 generations on:	
Temperature (°C)	Curing agent	Succinate	Methanol
30	None	<0.5	<0.5
32	None	22	99
30	Mitomycin C (1.0 mg ml ⁻¹)	4	<0.5

The data presented in Table 2 show that a high yield of Dcm^- mutants was obtained upon growth of strain DM4 at the supra-optimal temperature of 32 °C. The loss of DCM dehalogenase in these mutants as well as in the Dcm^- derivatives obtained by mitomycin C treatment appeared to be non-reversible (revertant frequency <10⁻⁹). Using the antibody assay, which is more sensitive than the DCM dehalogenase enzyme assay, we examined whether the Dcm^- mutants still produced DCM dehalogenase protein. As evident from the example presented in Fig. 2 none of the Dcm^- mutants produced antigenic material reacting with the antibody against DCM dehalogenase, whereas non-induced wild-type cells gave a strong signal. This suggests that the Dcm^- mutants are not defective in the regulation of DCM dehalogenase synthesis or in DCM dehalogenase activity but have lost the structural gene of this enzyme.

Fig. 3. Southern blot hybridization of ^{32}P -labelled pME1510 to *Hind*III/*Sal*I-digested total cellular DNA from the wild-type DM4 and from the *Dcm*⁻ mutant DM4-2cr. Lanes: 1, pVK100; 2, pME1510; 3, total cellular DNA from DM4; 4, total cellular DNA from DM4-2cr. Lanes 1 and 2 contained 0.2 µg DNA each and the film was exposed for 2 h. Lanes 3 and 4 contained 1.0 µg DNA at a film exposure time of 100 h.

All of the 100 *Dcm*⁻ mutants examined for their plasmid content had lost plasmid pME1511. In most cases this loss was accompanied by the appearance of novel plasmid bands whose restriction patterns differed from the restriction profiles of the wild-type plasmids. The complex profiles observed were presumably due to DNA rearrangements. Eight *Dcm*⁺ clones from populations which had undergone temperature curing were also analysed for their plasmid content and all of them contained an intact plasmid pME1511. A clear-cut change was observed in the *Dcm*⁻ strains DM4-2c and its *Sm*^r derivative DM4-2cr (Fig. 1, Table 1); both had lost pME1511 and retained plasmids pME1512 and pME1513. Restriction analysis confirmed the identity of plasmids pME1512 and pME1513 of the wild-type with the corresponding plasmids contained in strain DM4-2cr (see Fig. 4a). The properties of strain DM4-2cr made it unlikely that pME1512 or pME1513 encode DCM-utilization genes. Strain DM4-161 is a *Dcm*⁺ strain which had undergone temperature curing. It had lost pME1513 and retained plasmids pME1511 and pME1512 (Fig. 1), which demonstrated that pME1513 was not associated with the ability to grow on DCM. A second round of temperature curing with strain DM4-161 resulted in the

Table 3. Expression of DCM dehalogenase in different hosts

Strain	Growth conditions	DCM dehalogenase specific activity [mkat (kg protein) ⁻¹]	
		Non-induced*	Induced†
<i>Methylobacterium</i> sp. DM4		0.12	1.87
<i>Methylobacterium</i> sp. DM4-2cr		<0.02	<0.02
<i>Methylobacterium</i> sp. DM4-2cr(pME1510)		0.15	2.16
<i>Methylobacterium</i> sp. AM1r		<0.02	<0.02
<i>Methylobacterium</i> sp. AM1r(pME1510)		0.12	0.63
<i>E. coli</i> HB101(pVK100)		<0.02	<0.02
<i>E. coli</i> HB101(pME1510)		<0.02	<0.02

* *Methylobacterium* strains were grown on minimal medium methanol; *E. coli* HB101 was grown on LB medium.

† *Methylobacterium* strains were grown on minimal medium with methanol and DCM as growth substrates; *E. coli* HB101 was grown on LB medium containing 2 mM-DCM.

Dcm⁻ mutant DM4-161c. It contained pME1514, a 50 kb plasmid which, by restriction analysis, was shown to be unrelated to the wild-type plasmid pME1511 (data not shown). Taken together, the curing experiments suggested that the DCM-utilization character was correlated with the possession of an intact plasmid pME1511. To examine this possibility in more detail the DCM-utilization genes were cloned.

Cloning of a DNA fragment carrying the *dcm* region

A total of 1200 Tc^r transductants of *E. coli* HB101, obtained with *in-vitro*-packaged DNA from a *Hind*III cosmid-library of *Methylobacterium* sp. DM4, were tested for the production of DCM dehalogenase by the antibody assay. The transductants were grown under inducing conditions on nutrient agar plates in a DCM-containing atmosphere, but none of them gave a positive signal. Fifty transductants were examined for their plasmid content: all of them contained recombinant cosmids having in common a 23 kb *Hind*III fragment (pVK100) and one or more additional *Hind*III fragments corresponding to insert sizes in the range 15 to 26 kb with an average size of about 20 kb. Of the 1200 transductants 98.6% were Km^sTc^r indicating inserted DNA in the Km-resistance gene.

The cosmid clone bank was therefore mobilized with the help of plasmid pRK2013 into the Dcm⁻ mutant DM4-2cr. *Methylobacterium* transconjugants growing on methylamine agar with Sm and Tc were selected and screened on DCM indicator-plates for the ability to degrade DCM. Among the 1200 transductants tested by this procedure one led to DCM-degrading transconjugants. The 44 kb plasmid of this strain was designated pME1510. Restriction enzyme analysis demonstrated that pME1510 extracted from *E. coli* HB101 and from *Methylobacterium* sp. DM4-2cr were identical. To determine whether the DNA insert of pME1510 was of *Methylobacterium* origin, total genomic DNA of this organism was digested with *Hind*III and *Sal*I, and probed with nick-translated ³²P-labelled pME1510 (Fig. 3). The recombinant cosmid pME1510 showed strong homology with DNA of the wild-type strain DM4, whereas it did not hybridize with DNA of the Dcm⁻ mutant DM4-2cr. These observations demonstrate that the 21 kb *Hind*III fragment of pME1510 is of *Methylobacterium* DM4 origin and that this fragment is deleted from the genome of the Dcm⁻ mutant DM4-2cr.

Plasmid pME1510 was transferred with the help of pRK2013 from *E. coli* to a number of facultative methyltrophs unable to grow on DCM and all probably belonging to the genus *Methylobacterium* (Green & Bousfield, 1982). It conferred the ability to utilize DCM as the sole carbon and energy source to *Methylobacterium organophilum* XX, *Protaminobacter ruber*, *Pseudomonas* sp. M27, *Methylobacterium* sp. AM1 and *Pseudomonas extorquens*. With the exception of *Methylobacterium organophilum* XX, DCM degradation appeared to be stably maintained in these organisms. As shown in Table 3, pME1510 introduced in its

Fig. 4. Hybridization of ^{32}P -labelled pME1510 encoding the DCM dehalogenase gene from *Methylobacterium* sp. strain DM4 to *Hind*III-digested plasmid DNA from DM4. (a) Ethidium-bromide-stained gel; (b, c) autoradiograms of the corresponding Southern blots after hybridization with ^{32}P -labelled pME1510 (b) and pME1515 (c) as the probes. Lanes: 1, Plasmid DNA from the wild-type strain DM4; 2, plasmid DNA from the *Dcm*⁻ mutant DM4-2cr; 3, plasmid DNA from the recombinant clone DM4-2cr(pME1510). Restriction fragments indicated by the small arrows (a) originated from pME1511.

Methylobacterium hosts conferred a DCM dehalogenase specific activity which was comparable to the activity present in the wild-type strain DM4. Since the dehalogenase activity encoded by pME1510 was induced by DCM in *Methylobacterium* sp. AM1r, both the DCM dehalogenase structural gene and a putative regulatory gene appear to be located on the cloned fragment. The absence of detectable dehalogenase activity in extracts of *E. coli* HB101 carrying pME1510 is in accordance with the results of the antibody assay and is probably due to the complete lack of expression of the DCM dehalogenase gene in *E. coli*.

After growing *E. coli* HB101(pME1510) for 100 generations under non-selective conditions 61% of the population exhibited the Tc resistance conferred by the plasmid. When *Methylobacterium* DM4-2cr(pME1510) was grown for 30 generations on methanol without Tc 99% of the cells were Tc^r and *Dcm*⁺.

Chromosomal location of the dcm region

The curing experiments described above indicated that the DCM-utilization genes might be encoded on plasmid pME1511. We examined this possibility by using pME1510 as a hybridization probe against the purified plasmid preparation from *Methylobacterium* sp. DM4. Since the efficiency of DNA transfer in the Southern hybridization procedure decreases with increasing size of DNA fragments (Wahl *et al.*, 1979), it might be low for the entire 120 kb plasmid pME1511. We therefore used concentrated *Hind*III-restricted plasmid DNA of DM4 in the Southern hybridization experiment. As shown in Fig. 4(b) (lane 1) no hybridization signal corresponding to a 21 kb *Hind*III fragment could be detected. In a control experiment we used

pME1515, a recombinant plasmid of pVK100, with a 7 kb *Sal*I fragment of pME1511 as a hybridization probe. This probe produced definite signals at the position of pME1511 fragments (Fig. 4c). We thus conclude that the *dcm* genes are located neither on plasmid pME1511 nor on one of the two other cryptic plasmids of strain DM4, but must be carried on the chromosome or on a megaplasmid of *Methylobacterium* sp. DM4.

DISCUSSION

DCM is a xenobiotic compound which has been introduced into the environment in significant quantities by the industrial activities of the past forty years. In facultative methylotrophic bacteria utilizing this compound as a carbon source a high proportion of the total cellular protein consists of DCM dehalogenase, a catalytically inefficient, highly specific enzyme whose evolutionary origin is unknown (Kohler-Staub & Leisinger, 1985). The numerous examples of plasmids coding for peripheral reactions in the catabolism of xenobiotics by soil bacteria (Haas, 1983) gave rise to the question whether the DCM-utilization character was also associated with a degradative plasmid. We did three types of experiments to examine this possibility in *Methylobacterium* sp. DM4.

Firstly, we investigated the correlation of the plasmid pattern with the Dcm⁻ phenotype. Curing experiments showed that two of the three plasmids detected in the organism investigated were not associated with DCM utilization. However, loss of pME1511, the third and largest (120 kb) plasmid, was paralleled by the loss of the Dcm⁺ character. In a second series of experiments, not reported in the Results section, we attempted to demonstrate the concomitant transfer of DCM utilization and plasmid pME1511 from the wild-type strain to the cured derivative DM4-2cr. Transfer of the Dcm⁺ phenotype by conjugation was not achieved. Despite the successful use of the broad-host-range plasmid R68.45 (Haas & Holloway, 1978) for chromosome mobilization in *Methylobacterium* AM1 (Tatra & Goodwin, 1983) we failed to transfer the Dcm⁺ character by R68.45 to strain DM4-2cr. R-Prime plasmids conferring the Dcm⁺ phenotype could have been analysed with respect to the origin of their DNA. Since they were not found, these and other *in vivo* genetic experiments directed at determining the genomic location of the DCM-utilization genes have remained inconclusive. The goal of a third type of experiments was therefore to obtain direct evidence for the location of the *dcm* genes on either a plasmid or the chromosome. This was achieved by Southern hybridization experiments using a cloned 21 kb *Hind*III fragment encompassing the *dcm* genes as a probe. Since total genomic DNA of the wild-type strain DM4 hybridized with this probe, whereas a plasmid preparation from the same organism did not, the *dcm* genes must be encoded on the chromosome or on an extrachromosomal element that so far has remained undetected. No hybridization signal was obtained with total DNA from the cured derivative DM4-2cr.

As a consequence of this result we postulate that temperature curing and mitomycin C treatment not only lead to plasmid loss, but also induce the formation of a deletion larger than 21 kb and covering the *dcm* genes. Alternatively, curing might lead to the loss of an undetected megaplasmid.

An interesting question concerns the mechanism of transfer of the *dcm* genes between different methylotrophs. Since different isolates have been shown to contain closely similar if not identical DCM dehalogenases (Kohler-Staub *et al.*, 1986), this process is assumed to occur readily in the environment. Transposition of the *dcm* region onto broad-host-range plasmids thus may be an essential step in spreading the Dcm⁺ phenotype. Tentative evidence for the occurrence of transposons encoding haloalkanoic acid dehalogenase structural genes has been obtained in *Moraxella* (Kawasaki *et al.*, 1981) and in *Pseudomonas putida* PP3 (Slater *et al.*, 1985). The cloned *dcm* genes will be useful to investigate the evolution and distribution of the DCM utilization system in facultatively methylotrophic soil bacteria.

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