Association of newly discovered IS elements with the dichloromethane utilization genes of methylotrophic bacteria

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Dichloromethane (DCM) dehalogenases enable facultative methylotrophic bacteria to utilize DCM as sole carbon and energy source. DCM-degrading aerobic methylotrophic bacteria expressing a type A DCM dehalogenase were previously shown to share a conserved 4.2 kb *Bam*HI DNA fragment containing the dehalogenase structural gene, *dcmA*, and *dcmR*, the gene encoding a putative regulatory protein. Sequence analysis of a 10 kb DNA fragment including this region led to the identification of three types of insertion sequences identified as IS1354, IS1355 and IS1357, and also two ORFs, *orf353* and *orf192*, of unknown function. Two identical copies of element IS1354 flank the conserved 4.2 kb fragment as a direct repeat. The occurrence of these newly identified IS elements was shown to be limited to DCM-utilizing methylotrophs containing a type A DCM dehalogenase. The organization of the corresponding *dcm* regions in 12 DCM-utilizing strains was examined by hybridization analysis using IS-specific probes. Six different groups could be defined on the basis of the occurrence, position and copy number of IS sequences. All groups shared a conserved 5.6 kb core region with *dcmA*, *dcmR*, *orf353* and *orf192* as well as IS1357. One group of strains including *Pseudomonas* sp. DM1 contained two copies of this conserved core region. The high degree of sequence conservation observed within the genomic region responsible for DCM utilization and the occurrence of clusters of insertion sequences in the vicinity of the *dcm* genes suggest that a transposon is involved in the horizontal transfer of the DCM-utilization character among methylotrophic bacteria.

Keywords: dichloromethane, dehalogenase, IS element, methylotrophic bacteria

INTRODUCTION

Some Gram-negative facultative methylotrophic bacteria utilize dichloromethane (DCM) as the sole carbon and energy source. The initial step of DCM utilization is catalysed by DCM dehalogenase, an enzyme that transforms DCM to inorganic chloride and formaldehyde, a central metabolite of methylotrophic growth. Functionally and by their amino acid sequence, bacterial DCM dehalogenases belong to the Theta-class of the large and ubiquitous superfamily of glutathione S-transferases (Leisinger *et al.*, 1994).

Bacterial DCM dehalogenases have been shown to fall into two classes, type A and type B, which significantly differ from each other with respect to kinetic properties and amino acid sequence (Scholtz *et al.*, 1988; Bader & Leisinger, 1994). In *Methylobacterium* sp. strain DM4 and other methylotrophs with a type A enzyme, the dehalogenase structural gene, *dcmA*, and *dcmR*, the gene encoding a regulatory protein governing dehalogenase expression (La Roche & Leisinger, 1990, 1991), are located on a conserved 4.2 kb *Bam*HI fragment. In some strains, this fragment is carried on a 120 kb plasmid, whereas in others it is located on the chromosome (Leisinger *et al.*, 1994). These findings and the observation that the ability of bacteria to utilize DCM is readily lost under non-selective conditions (Galli & Leisinger, 1988) suggest that the *dcm* genes have been distributed among facultative methylotrophs by hori-

Abbreviations: DCM, dichloromethane; DIG, digoxigenin.

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zontal gene transfer. To explore this possibility in more detail, we have analysed in *Methylobacterium* sp. strain DM4 the DNA regions flanking the *dcm* genes. Here we report that sequence similarity between a number of methylotrophs with a type A DCM dehalogenase extends over 10 kb. This conserved region comprises, in addition to *dcmA* and *dcmR*, two ORFs of unknown function as well as an assortment of newly discovered insertion sequences that appear to be specifically associated with the DCM utilization genes.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1.

Methylotrophic bacteria were grown at 30 °C in minimal medium with methanol or DCM as carbon sources as described elsewhere (Kohler-Staub *et al.*, 1986). *Escherichia coli* strains were grown on LB medium (Miller, 1972) at 37 °C. Antibiotics for selection were added at the following concentrations: 200 µg ampicillin ml⁻¹, 25 µg tetracycline ml⁻¹.

DNA manipulations. General genetic techniques were performed according to standard protocols (Ausubel *et al.*, 1997; Sambrook *et al.*, 1989). Plasmids were introduced into *E. coli* strains following the procedure described by Inoue *et al.* (1990).

Southern blot analysis. Digested genomic DNA was loaded on 1% agarose gels. The 1 kb DNA ladder used as molecular mass marker was labelled with digoxigenin (DIG)-dUTP. The 3' ends of the marker DNA were degraded by exonuclease

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant phenotype*/genotype	Source/reference
<i>Methylobacterium</i>		
<i>Methylobacterium extorquens</i> AM1	Dcm ⁻	Green (1992)
<i>Methylobacterium organophilum</i> XX	Dcm ⁻	Green (1992)
<i>Methylobacterium</i> sp. DM4	Dcm ⁺	Gälli & Leisinger (1985)
<i>Methylobacterium</i> sp. DM4-2cr	Dcm ⁻	Gälli & Leisinger (1988)
<i>Methylobacterium</i> sp. CM4	Dcm ⁻	Doronina <i>et al.</i> (1996)
<i>Hyphomicrobium</i>		
<i>Hyphomicrobium</i> sp. GJ21	Dcm ⁺	D. Janssen, University of Groningen, Netherlands
<i>Hyphomicrobium</i> sp. DM2	Dcm ⁺	Stucki <i>et al.</i> (1981)
<i>Methylophilus</i>		
<i>Methylophilus</i> sp. DM11	Dcm ⁺	Scholtz <i>et al.</i> (1988)
<i>Pseudomonas</i>		
<i>Pseudomonas</i> sp. DM1	Dcm ⁺	Brunner <i>et al.</i> (1980)
<i>Methylophabundus</i>		
<i>Methylophabundus multivorans</i> DM13	Dcm ⁺	Doronina <i>et al.</i> (1995)
Unidentified methylotrophs		
Strains DM3, DM5-10	Dcm ⁺	Gälli & Leisinger (1985), Gälli (1986)
<i>E. coli</i>		
DH5α	<i>supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 relA1</i>	Gibco-BRL
S17-1	<i>hsdR RP4-2 kan::Tn5 tet::MU</i> chromosomally integrated	Simon <i>et al.</i> (1983)
Plasmids		
pBluescript KS(+)	Ap ^r	Stratagene
pSUP202	Ap ^r , <i>oriT</i> from RP4	Simon <i>et al.</i> (1983)
pME1510	Tc ^r , Dcm ⁺ , (pVK100), 21 kb <i>Hind</i> III fragment encompassing the <i>Methylobacterium</i> sp. DM4 <i>dcm</i> region	Gälli & Leisinger (1988)
pME1540	Ap ^r , (pBLS+), 1.5 kb <i>Hind</i> III- <i>Pst</i> I fragment with <i>dcmA</i>	S. La Roche, unpublished
pME1839	Ap ^r , (pBLS+), 1.1 kb <i>Eco</i> RI- <i>Sac</i> I fragment of IS1354 _L	Schmid-Appert (1996)
pME1841	Ap ^r , (pBLS+), 2.7 kb <i>Sac</i> I- <i>Bam</i> HI fragment with IS1355 and IS1357	Schmid-Appert (1996)
pME1874	Ap ^r , (pSUP202), 21 kb <i>Hind</i> III fragment of pME1510	Schmid-Appert (1996)
pME1887	Ap ^r , (pBLS+), 0.4 kb <i>Eco</i> RV fragment of IS1357	Schmid-Appert (1996)

*Dcm⁺, DCM-utilizing; Dcm⁻, unable to utilize DCM.

Fig. 1. Physical map of the *dcm* region of *Methylobacterium* sp. DM4. The genes *dcmA* and *dcmR* as well as the ORFs *orf353* (pos. 2510–3538 in M32346) and *orf192* (pos. 3573–4151 in M32346) are shown as white boxes. Insertion sequences are shown as hatched boxes. Arrows above ORFs indicate the direction of transcription of the corresponding coding regions. Black triangles at the ends of the boxes representing IS1355 and IS1354 sequences indicate the presence of inverted repeats, and the direct repeats found in the nucleotide sequences flanking IS1354_L and IS1354_R are shown as short arrows (see Fig. 2).

before labelling. For this purpose 10 µg 1 kb DNA (Gibco BRL) were treated with 40 units T4 DNA polymerase for 2 min at 37 °C in 20 µl 53 mM Tris acetate (pH 7.9), 66 mM sodium acetate, 10 mM MgCl₂, 0.5 mM DTT containing 0.1 mg BSA ml⁻¹. The re-synthesis reaction was carried out for 25 min at 37 °C after addition of DIG-dUTP DNA-labelling mix (Boehringer). After electrophoresis, DNA was denatured and blotted on Hybond-N membranes (Amersham) as described by the manufacturer. DNA hybridization probes were labelled by PCR amplification with alkali-labile DIG-dUTP nucleotide mix. Specific DNA hybridization probes were obtained by standard protocols using 100 pmol each primer and 3 fmol template DNA. The pBluescript KS(+) derivatives pME1839, pME1841, pME1887 and pME1540, carrying appropriate sequences from IS1354, IS1355, IS1357 and *dcmA* (Table 1), were used as DNA templates in the PCR reaction. The primers used to obtain specific DNA probes had the following nucleotide sequences (nucleotide positions in brackets refer to the sequence shown in Fig. 1): 5'-CGACGGCAACACGGT-3' (212–226), and 5'-GAGGGCA-TGACGACGGAATGGAAG-3' (1042–1019) for IS1354; 5'-ACGGCACATTGGAGCGAA-3' (1772–1789) and 5'-ATAA-AAGGCAACTGTAGT-3' (2390–2373) for IS1355; and universal primers 5'-ATTAACCCTCACTAAAGG-3' (T3) and 5'-AATACGACTCACTATAGG-3' (T7) for IS1357 and *dcmA*, respectively. Hybridization at 68 °C and subsequent immunochromatological detection was performed following the procedure described by Engler-Blum *et al.* (1993). Probes were stripped from the membrane by alkali treatment for repeated hybridization of the same blot with probes of different specificity as described by the manufacturer.

DNA sequencing and sequence analysis. Fragments of the cosmid pME1510 were subcloned into pBluescript KS(+) for sequence analysis. DNA sequences were determined from double-stranded pBluescript subclones according to a modified chain-termination method (Schuurman & Keulen, 1991). Nucleic acid and amino acid sequences were analysed by using the Genetics Computer Group program package, version 8.1 (University of Wisconsin, Madison, WI, USA). DNA and amino acid sequence similarity searches of non-redundant DNA and protein sequence databases were performed at the NCBI using the BLAST network service (Altschul *et al.*, 1990) and the FASTA program, version 2.0.2 (Pearson, 1996). Sequence alignments were done with the GCG program PILEUP and identity scores were obtained from identity matrices of multiple sequence alignments calculated with the GCG program OLDDISTANCES. IS1354, IS1355 and IS1357 are

Plasmid Reference Centre listings (E. Lederberg, Stanford, CA, USA).

Mobilization of the *dcm* region into methylotrophs unable to utilize DCM. Exponentially growing cultures of donor (*E. coli* S17-1 containing plasmid pME1874) and recipient (*Methylobacterium extorquens* AM1, *Methylobacterium organophilum* XX, and *Methylobacterium* sp. CM4) were concentrated 40-fold, mixed in a 1:3 ratio, spotted onto LB agar and cocultivated at 30 °C for 24 h. Selection of transconjugants was carried out by streaking out onto minimal agar using DCM as sole carbon source.

RESULTS

Sequence analysis of the *Methylobacterium* sp. DM4 *dcm* region

The *dcm* genes of *Methylobacterium* sp. DM4 are located on a 4.2 kb chromosomal *Bam*HI restriction fragment (La Roche & Leisinger, 1990; Leisinger *et al.*, 1994). A more detailed characterization of this region in *Methylobacterium* sp. DM4 and in *Hyphomicrobium* sp. DM2 by Southern analysis indicated that similarity between these strains extended to at least 10 kb, and the corresponding region was cloned and sequenced from strain DM4. Fig. 1 gives an overview of the conserved region and indicates the location of newly sequenced DNA fragments. The region downstream of *dcmA* features two ORFs, *orf353* and *orf192*, and IS1354. A second identical copy of this element is located at the left-hand border of the sequenced *dcm* region. IS1355 is found immediately adjacent to this element and is in turn followed by IS1357. The nucleotide sequence of the DNA fragment comprising the three newly identified insertion sequences is shown in Fig. 2, and the structural properties of these elements are summarized in Table 2.

The gene *orf353* located downstream (3') of *dcmA* (Fig. 1) encodes a putative protein with a molecular mass of 40 kDa. Its sequence was preceded by a putative RBS but lacked sequences with similarity to *E. coli* consensus promoters or *Methylobacterium* promoter motifs. A database search for similar proteins gave 45% identity of Orf353 with OrfA, the deduced product of an

Fig. 2. Single strand nucleotide sequence of IS1354_L, IS1355 and IS1357 (accession no. X96995). IS1354_R (pos. 4197–5627 in M32346), the second copy of IS1354 located downstream of *orf192* in strain DM4, is identical to IS1354_L. Inverted repeats (IR) and direct repeats (DR) at both ends of an insertion sequence are indicated by arrows below and above the sequence, respectively. Translational products of the putative transposase genes are given below the nucleotide sequence. Stop codons are indicated by asterisks. Potential RBSs are in bold. Identities to the –35/–10 promoter consensus of *E. coli* are indicated by dots. The putative frameshift signal in IS1355 is indicated by lines above and below the sequence. Boxed nucleotides accompanied by lettered numerals depict a possible pseudoknot RNA secondary structure downstream of the putative frameshift signal (Farabaugh, 1996; see text). Residues in bold in Orf153 and Orf179 indicate the potential translation frameshifting site leading to the Orf153–Orf179 fusion protein. Underlined residues in the sequence of Orf421 correspond to the D-(3)-G-(HVMF)-(6)-(STAV)-(LIVMFVW)-(PT)-(1)-(STAV)-(2)-Q-R-C-(2)-H 'mutator' motif (Eisen *et al.*, 1994) found in all IS1354-related transposases.

Table 2. Insertion sequences IS1354, IS1355 and IS1357 of *Methylobacterium* sp. strain DM4

IS element	Length (bp)	Transposase	Inverted repeat (bp)	Direct repeat (bp)
IS1354	1431	ORF421	48	8
IS1355	975	ORF153 ORF179	9–10	ND*
IS1357	ND*	ORF411	ND*	ND*

*ND, Not determined. The length of these sequences could not be determined, presumably due to sequence overlap between IS1354 and IS1355 and between IS1355 and IS1357.

incomplete ORF downstream (3') of the *dcmA* gene of *Methylophilus* sp. strain DM11 (Bader & Leisinger, 1994). This suggests that the clustering of *dcmA* with *orf353* is an evolutionarily conserved trait, but the function of the proteins encoded by *orf353* or *orfA* remains obscure. Deletion of *orf353* had no effect on DCM utilization (Schmid-Appert, 1996) so these genes do not appear to be involved in DCM metabolism. A modest level of 27% amino acid identity was also noted between Orf353 and protein P8 of *Nocardia lactam-durans*. Protein P8 is one of the proteins implicated in converting cephalosporin C to 7-methoxycephalosporin C (Coque *et al.*, 1995), but it is unclear if this reaction relates in any way to dihalomethane metabolism.

A second ORF, *orf192*, was also found downstream of *orf353*. The corresponding 21 kDa protein sequence showed no significant identity to known sequences. As for *orf353*, a putative RBS was detected in the region immediately upstream of this reading frame but no promoter sequences could be found.

Characteristics of IS1354

IS1354 extended over 1431 bp and carried 48 bp imperfect terminal inverted repeats which were flanked by 8 bp direct repeats. These flanking sequence repetitions, 5'-TTGAATCA-3' for IS1354_L and 5'-GCCGTCAA-3' for IS1354_R, were not identical for the two copies of the element. The major ORF of IS1354 encoded a putative protein of 421 amino acids, termed Orf421. It was preceded by a putative -35/-10 promoter region and a potential RBS (Fig. 2). Orf421 exhibited similarity to putative transposases of insertion sequences from both Gram-positive and Gram-negative bacteria (Table 3). Sequence alignments of Orf421 to related transposases (not shown) led to the identification of a conserved C-terminal motif corresponding to the consensus sequence described by Eisen *et al.* (1994) for a family of insertion sequences including elements of plants and bacteria (Fig. 2). The size of IS1354-related elements described in the literature ranges from 1.3 to 1.7 kb. Upon insertion, they give rise to 7–9 bp direct repeats of the target site, and carry 15–41 bp inverted repeats at their extremities. Like IS1354, these elements contain a single ORF encoding a potential transposase composed of between 296 and 474 amino acids. Most of the insertion sequences hom-

ologous to IS1354 are present as multiple copies in their host strains. No sequence similarities between the target sites of these copies have been observed so that insertion of these elements is thought to be non-specific (Guilhot *et al.*, 1992; Yates *et al.*, 1988).

Characteristics of IS1355

IS1355 was 975 bp in length and had 9 and 10 bp terminal inverted repeats. No sequence duplications were observed in the flanking DNA. This was possibly caused by the subsequent insertion of IS1354 next to IS1355 since the 9 bp inverted repeat at the left end of IS1355 was identified within the 8 bp direct repeat flanking IS1354 (Fig. 2).

The nucleotide sequence of IS1355 revealed a short ORF, *orf153*, between nucleotides 61 and 520 in IS1355. It was partly overlapped by *orf179*, which was shifted in phase of translation by -1 nucleotide and extended from nucleotide positions 380–897 in IS1355. While *orf153* was preceded by a potential -10/-35 promoter and by a putative RBS (Fig. 2), none of these structures was observed in the region 5' of *orf179*. Sequences similar to both Orf153 and Orf179 were found within a subgroup of the large IS5/IS4 family of insertion sequences (Rezsöházy *et al.*, 1993). Similar transposase sequences in this group are also often encoded by two overlapping ORFs (Rezsöházy *et al.*, 1993). The sequence 5'-AAAAAGGGGG-3' found in the region where *orf153* and *orf179* overlap (Fig. 2) was very similar to potential ribosomal frameshifting signal sequences described by Iversen *et al.* (1994) for IS1355-related IS elements. These signals are known to direct the formation of fusion proteins as the result of -1 translational frameshifting with simultaneous slippage (Chandler & Fayet, 1993; Farabaugh, 1996). Accordingly, a frameshifting event at position 1894 of the IS1355 nucleotide sequence (Fig. 2) would result in a hypothetical fusion protein Orf153-Orf179 with a higher degree of identity to IS1355-related transposases encoded by one single reading frame than the polypeptides derived separately from *orf153* and *orf179* (Table 3). Interestingly, the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803, the sequence of which was recently reported (Kaneko *et al.*,

Table 3. Similarity of IS1354, IS1355 and IS1357 of *Methylobacterium* sp. DM4 to other insertion sequences

Organism	Insertion sequence			Putative transposases of <i>Methylobacterium</i> sp. DM4 IS elements (percentage amino acid identity)				
	Element	Reference	Protein acc. no.*	IS1354	IS1355		IS1357	
				Orf421	Orf153	Orf179	Orf153– Orf179†	Orf411
<i>Clostridium perfringens</i>	IS1151	Daube <i>et al.</i> (1993)	S31644	27.1				
<i>Mycobacterium smegmatis</i>	IS6120	Guilhot <i>et al.</i> (1992)	P35883	26.3				
<i>Sinorhizobium meliloti</i>	ISRM5	Laberge <i>et al.</i> (1995)	Q52873	25.6				
<i>Burkholderia cepacia</i>	IS406	Wood <i>et al.</i> (1991)	P24575	25.5				
<i>Streptococcus thermophilus</i>	IS1191	Guédon <i>et al.</i> (1995)	Q56104	24.3				
<i>Mycobacterium avium</i>	IS1245	Guerrero <i>et al.</i> (1995)	Q48368	24.2				
<i>Acetobacter xylinum</i>	IS1032	Iversen <i>et al.</i> (1994)	Q44566		40.3	48.9	51.6	
<i>Acetobacter xylinum</i>	IS1031c	Coucheron (1993)	Q44579		44.2	44.4	50.9	
<i>Sinorhizobium meliloti</i>	ISRM4	Soto <i>et al.</i> (1992)	Q52958‡(X65471)		46.8	40.6	49.8§	
<i>Synechocystis</i> sp. PCC 6803	HSII1791/1792§	Kaneko <i>et al.</i> (1996)	(D90905)		45.0	37.1	41.9	
<i>Synechocystis</i> sp. PCC 6803	Slr0350§¶	Kaneko <i>et al.</i> (1996)	Q57495		35.1	33.3	40.5	
<i>Synechocystis</i> sp. PCC 6803	Slr0799/0800§	Kaneko <i>et al.</i> (1996)	Q55944/Q55945		45.4	35.4	40.0§	
<i>Streptomyces lividans</i>	Tn4811	Chen <i>et al.</i> (1992)	Q54404		33.8	32.8	37.6	
<i>Burkholderia cepacia</i>	IS402§	Ferrante & Lessie (1991)	P24537/P24536		21.1	18.9	22.4§	
<i>Agrobacterium tumefaciens</i>	IS427§	De Meirsmen <i>et al.</i> (1990)	Q44425/PQ0496		22.5	18.0	21.9§	
<i>Synechocystis</i> sp. PCC 6893	HSII0667	Kaneko <i>et al.</i> (1996)	Q55974					24.7
<i>Synechocystis</i> sp. PCC 6803	HSII0092	Kaneko <i>et al.</i> (1996)	Q55893					21.0
<i>Synechocystis</i> sp. PCC 6803	Slr0099	Kaneko <i>et al.</i> (1996)	Q55871					20.6
<i>Calothrix</i> sp. PCC 7601	IS701	Mazel <i>et al.</i> (1991)	Q00461					19.0

* Protein database accession numbers; if not available, the gene entry accession number is given in parentheses.

† The hypothetical fusion protein Orf153–Orf179 results from a –1 translation frameshifting event with simultaneous slippage at position 1894 of the IS1355 nucleotide sequence (see text and Fig. 3).

‡ The ISRM4 sequence used here was proposed by Iversen *et al.* (1994). The introduction of a 1 nucleotide gap yields one continuous ORF with overall higher similarity to this group of transposase sequences.

§ Putative transposases related to IS1355 which appear to be encoding by two overlapping ORFs. Observed putative frameshift sequences: AAAAAAGGGGG (HSII1791/HSII1792, Slr0799/slr0800), AAAAAGGGGG (IS402), AAAAAAGGGGG (IS427).

|| Putative transposases of *Synechocystis* as annotated by Kaneko *et al.* (1996). The presence of insertion sequence structures enclosing the corresponding ORFs remains to be demonstrated.

¶ Four other identical putative transposase sequences and at least six further sequences differing only in a few amino acids are found in the *Synechocystis* sp. PCC 6803 genome.

1996), features many genes encoding putative transposase proteins similar to *orf153* and *orf179*, both as single full-length ORFs and as overlapping ORFs. With

regard to the latter, very similar putative frameshift signals to that found in IS1355 were found in at least two other cases (see Table 3).

Fig. 3. Detection of IS1354, IS1355 and IS1357 in total DNA of various DCM-utilizing methylotrophs. The same membrane was hybridized in turn with DIG-labelled probes specific for each insertion element. (a) Southern analysis of *Sal*I-digested total DNA from strain DM1 (lane a), DM2 (lane b), DM4 (lane c), DM5 (lane d), DM11 (lane e), DM13 (lane f), GJ21 (lane g) with DIG-labelled probes of IS1354, IS1355 and IS1357. Fragment sizes of the DNA marker are indicated in kb. (b) Schematic representation of the specific probes used for hybridization. IS1354, IS1355 and IS1357 are shown as hatched boxes. Black bars below the boxes depict the size and position of the IS-specific probes used to detect IS1354, IS1355 and IS1357, respectively. Element-internal *Sal*I sites (S) are indicated.

IS1357, an incomplete insertion sequence

IS1357, the third insertion sequence in the *dcm* region of *Methylobacterium* sp. DM4, was located adjacent to the right hand end of IS1355 (Fig. 2). The nucleotide sequence of this element contained *orf411*, an ORF whose 5' region lacks a potential promoter structure and RBS. The polypeptide potentially encoded by *orf411* exhibited a rather low level of identity of about 20% with putative transposases found in cyanobacteria (Table 3). Since the start of the *orf411* sequence overlapped with the right-hand extremity of IS1355, the product of *orf411* may have been truncated by insertion of IS1355 into IS1357. The fact that it was not possible to identify the ends of IS1357 would be consistent with such an insertion event.

Occurrence of the novel insertion sequences in methylotrophic bacteria

The distribution of the newly discovered insertion sequences among various methylotrophic bacteria was examined by Southern hybridization with the probes indicated in Fig. 3(b). The 12 DCM-utilizing methylotrophs with type A DCM dehalogenases contained copies of at least two different insertion sequences. In contrast, no hybridization signal was detected in total DNA of two *Methylobacterium* strains unable to grow with DCM, or in DNA of *Methylobacterium* sp. strain DM4-2cr (Gälli & Leisinger, 1988; Table 1), a temperature-cured mutant of strain DM4 which has suffered a deletion of the entire *dcm* region (data not shown). The three insertion sequences were also absent

from DNA of *Methylophilus* sp. strain DM11, an organism expressing a type B DCM dehalogenase (Fig. 3).

Organizational patterns of the *dcm* region in DCM-utilizing bacteria

Southern analysis under high stringency conditions was used to examine in more detail the arrangement of the IS elements and the *dcm* genes on the genomes of the DCM-utilizing bacteria with a type A dehalogenase. An example of a hybridization experiment with *SalI*-digested total DNA of representative strains is shown in Fig. 3. Similar experiments were done with DNA that had been digested with *Bam*HI, *Bgl*II, *Eco*RI and *Pst*I (data not shown). *SalI*-digested DNA of strains DM1, DM3, DM6, DM9 and DM10, as well as DM5, DM7 and DM8, revealed identical hybridization patterns, and only representative hybridizations with DNA of strains DM1 and DM5 are presented (Fig. 3a, lanes a and d). In the case of *SalI*-digested DNA of strain DM1, the relatively strong hybridization signals obtained at about 0.8 kb with the IS1354 probe, and at about 1.3 kb with the IS1355 and IS1357 probes, respectively, suggested the presence of two hybridizing fragments of identical size in strains of the DM1 class. Additional hybridization experiments with *Eco*RI and *Bgl*II-digested DNA then confirmed the presence in two copies of all three IS elements in strains of this class (data not shown). It thus became clear that strains DM1, DM3, DM6, DM9 and DM10 contained two copies of the *dcm* region. Fig. 4 presents a hybridization experiment of *Bgl*II- and *Pst*I-digested DNA carried out to unambiguously identify the two copies of the DCM dehalogenase gene in strain DM1. Since the *dcm* region had no restriction site for these enzymes, a single hybridizing band was expected, and found, for each copy of the *dcm* gene region.

Further, the larger number of hybridizing bands observed in the DNA of strains DM2 and DM13 with IS1354 and IS1357 probes, respectively, indicated that extra copies of these elements were present in these strains (Fig. 3a, lanes b and f). Subsequent Southern analysis suggested that such copies were located outside the *dcm* region (data not shown). Finally, the weak additional hybridization signals observed with the IS1355-specific probe in total DNA of strains DM2, DM4, DM13 and GJ21 (Fig. 3a, lanes b, c, f and g) suggested that more divergent IS1355-like sequences were also present in these strains.

The hybridization data were used to construct physical maps of the *dcm* region in the different strains, and these are shown in Fig. 5. In summary, six different patterns of organization were observed. The minimal structure present in all strains consisted of a conserved core region of 5.6 kb, which contains the genes *dcmR*, *dcmA*, *orf353* and *orf192* as well as IS1357. The organizational classes differ from each other with respect to the copy number and the location of additional insertion sequences flanking the conserved core region.

Fig. 4. Detection of a second copy of the conserved core region in *Pseudomonas* sp. DM1 and DM3. Southern analysis with a DIG-labelled probe of *dcmA* of total DNA of strain DM1 (lanes a and b) and DM3 (lanes c and d) digested with *Pst*I (lanes a and c) and *Bgl*II (lanes b and d), respectively.

Investigation of the transpositional activity of the *dcm* region

The 21 kb *Hind*III fragment of strain DM4 including the 10 kb *dcm* region defined in the present work was cloned into the suicide vector pSUP202 (Simon *et al.*, 1983; see Table 1), and mobilization of the resulting recombinant plasmid pME1874 into several *Methylobacterium dcm* strains was attempted. In no instance, however, could transconjugants be obtained that displayed the DCM-utilization phenotype, indicating the inability of the *dcm* region to transpose under the conditions of the experiment (M. Schmid-Appert & D. Gisi, unpublished results).

DISCUSSION

The three insertion sequences IS1354, IS1355 and IS1357 of *Methylobacterium* sp. strain DM4 can be ascribed to class I of transposable elements (Kleckner, 1981), but clearly belong to different groups on the basis of their sequence. A close association of three different insertion sequences on a short segment of DNA, as in the present case, has rarely been observed. However, many genes encoding putative transposases similar to IS1357 and especially to IS1355 of strain DM4 (Table 3) were found in the cyanobacterium *Synechocystis* sp. PCC 6803. In one instance at least, ORFs in the *Synechocystis* genome (ORFs sll0665–sll066) encoding polypeptides similar to those encoded by IS1355 were located immediately adjacent to an ORF (sll0667, see Table 3) similar to the C-terminal end only of Orf411 encoded by IS1357. This suggests that in *Synechocystis* sp. PCC 6803, an element similar to IS1355 inserted into a sequence similar to IS1357.

The observed accumulation of insertion sequences in the gene region involved in DCM degradation is suggestive

Fig. 5. Genetic organization of the *dcm* region in DCM-utilizing methylotrophs with a type A DCM dehalogenase. Insertion sequences are shown as hatched boxes. Black triangles indicate the inverted repeats at the ends of IS1354 and IS1355. *dcmA*, *dcmR*, *orf353* and *orf192* are shown as white arrows. The position of *SalI* sites (S) are indicated.

of a functional association between both types of genes. In addition to their ability to self-transpose during translocation to a foreign genetic context, IS elements can also play a role in gene activation (Galas & Chandler, 1989). In the present case, activation of *dcm* gene expression mediated by a promoter or regulation element internal to the insertion sequence is unlikely, since the upstream sequences of *dcm* genes are separated from the insertion sequences by the *dcm* genes themselves (Fig. 1).

Evidence for the location of specific catabolic genes on transposons is available for a number of systems (reviewed by Van der Meer *et al.*, 1992; Wyndham *et al.*, 1994), including several examples where the catabolic genes propagated by these elements encode dehalogenating enzymes. The chlorobenzene transposon Tn5280 of *Pseudomonas* sp. strain P51 is a typical representative of a class I composite transposon. It contains a bacterial dehalogenase gene flanked by two identical copies of an insertion sequence (Van der Meer *et al.*, 1991). Nevertheless, flanking insertion sequences do not always lead to mobility of the associated catabolic genes. For example, the DNA fragment containing the genes involved in 2,4,5-trichlorophenoxyacetic acid in *Burkholderia* (*Pseudomonas*) *cepacia* AC1100 were flanked by two copies of IS931, suggesting a possible class I composite transposon structure. The corresponding DNA fragment, however, could not be demonstrated

to be mobile (Haugland *et al.*, 1990). More unusual types of mobile genetic elements involved in dehalogenation processes have also been described. These include the *DEH* element of *Pseudomonas putida* PP3 and a mobile DNA segment of *Xanthobacter autotrophicus* GJ10 that contains IS1247. Both elements are associated with a gene encoding a haloacid dehalogenase (Thomas *et al.*, 1992; van der Ploeg *et al.*, 1995), and they transpose as genetic units of variable length. Whereas the structure of the *DEH* element remains ill-defined, that of strain GJ10 was shown to involve only one flanking copy of IS1247.

The occurrence of two identical copies of IS1354 directly flanking genes involved in DCM utilization in strain *Methylobacterium* sp. strain DM4 suggested at first that the genes spanned by the two copies, including the two insertion sequences IS1355 and IS1357, represent a class I composite transposon. Alternatively, the gene arrangement observed in the *dcm* region of DM4 may have been produced by cointegrate formation or by homologous recombination (Galas & Chandler, 1989). The presence of different target site duplications flanking the two copies of IS1354, however, argues against such events having taken place in the genome of DM4. Indeed, not all the DCM-utilizing strains which have been characterized contain two copies of IS1354 (Fig. 5). Of course, the analysis of the arrangement of insertion sequences in the *dcm* region of *Methylobacterium* sp.

strain DM4 and of other related methylotrophic DCM utilizers cannot by itself explain the mechanism by which the *dcm* region was acquired and distributed among methylotrophs. Experiments to observe or induce the transposition of the *dcm* region have not been successful so far. Nevertheless, the occurrence in many DCM-utilizing methylotrophs of a highly conserved DNA fragment featuring three different insertion elements flanking a core region including the *dcmA* gene (Fig. 5) strongly supports the idea that the *dcm* region was transposed in the past.

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