Identification of *dcmR*, the Regulatory Gene Governing Expression of Dichloromethane Dehalogenase in *Methylobacterium* sp. Strain DM4

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The genes for dichloromethane utilization by *Methylobacterium* sp. strain DM4 are encoded on a 2.8-kb sequenced DNA fragment, the *dcm* region. This fragment contains *dcmA*, the structural gene of dichloromethane dehalogenase and, upstream of *dcmA*, a 1.5-kb region responsible for inducibility of dichloromethane dehalogenase by dichloromethane. A fragment of the *dcm* region covering *dcmA* and 230 bp of its upstream region was integrated into the chromosome of a *Methylobacterium* sp. strain DM4 mutant deleted for the *dcm* region. This yielded a strain expressing dichloromethane dehalogenase constitutively at the induced level. Plasmids carrying various segments of the 1.5-kb regulatory region were tested for their ability to restore regulation. The data obtained led to the identification of *dcmR*, the structural gene of a putative *dcm*-specific repressor. Transcription of *dcmR* was divergent from *dcmA*. *dcmR* encoded a 30-kDa protein with a helix-turn-helix motif near the amino terminus. The transcription start sites of *dcmA* and *dcmR* were identified by nuclease S1 mapping. The promoter regions of these genes contained nearly identical 12-bp sequences covering positions −14 to −25 relative to the mRNA start sites. Experiments with *dcmR'-'lacZ* fusions demonstrated that *dcmR* expression was markedly autoregulated at the level of transcription and less so at the protein level. These findings are compatible with both *dcmA* and *dcmR* expression being negatively controlled at the transcriptional level by the DcmR protein.

Aerobic methylotrophic bacteria utilizing dichloromethane (DCM) as the sole carbon and energy source possess the enzyme DCM dehalogenase (18, 26). This enzyme converts DCM to formaldehyde and inorganic chloride in a glutathione-dependent reaction (20). In all of the aerobic methylotrophs tested, the catalytic activity of DCM dehalogenase is low and the enzyme is 50- to 80-fold induced by DCM (18, 26). The genes responsible for DCM utilization have been examined in *Methylobacterium* sp. strain DM4, a pink-pigmented facultative methylotrophic bacterium. They are encoded on a 2.8-kb *Bam*HI-*Pst*I DNA fragment located on the chromosome or on an undetected megaplasmid of this organism (19), and three cryptic plasmids carried by strain DM4 are unrelated to DCM metabolism (10). A 0.9-kb segment of the cloned and sequenced 2.8-kb *Bam*HI-*Pst*I fragment encodes *dcmA*, the structural gene of DCM dehalogenase. *dcmA* has been shown to belong to the glutathione S-transferase supergene family. DCM dehalogenase expression is subject to negative control at the transcriptional level, and our preliminary observations suggested that a regulatory protein responsible for this control is also encoded on the sequenced fragment, in the upstream region of *dcmA* (19).

In this communication, we report the functional analysis of the 1.5-kb region upstream of *dcmA*. We present evidence that this region contains *dcmR*, the structural gene of a *trans*-active protein controlling *dcmA* expression as well as its own synthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacteria and plasmids used in this study are listed in Table 1. Growth conditions for *Escherichia coli* and *Methylobacterium* strains were as described previously (19).

Construction of plasmids. Relevant plasmids (see Fig. 2) were constructed in *E. coli* S17-1, by using multiple linkers, with DNA fragments of the cloned 2.8-kb *dcm* region from plasmid pME1537. Vectors and important constructs used for cloning are listed in Table 1. Plasmid pME3048, a mobilizable ColE1 replicon derived from plasmid pRZ102 (15), was constructed by Voisard (30). Deletion of the 4.4-kb *Sma*I fragment of pRZ102 yielded pME3041. The *Eco*RI-*Hin*dIII fragment of this construct was replaced by an *Eco*RI-*Hin*dIII fragment containing a polylinker from pUC18 and, thus, resulting in pME3048 (30).

Plasmids pME1563 and pME1564, two integration vectors for *Methylobacterium* sp. strain DM4 carrying all or part of the *dcm* region, were constructed by using a 1.4-kb randomly cloned chromosomal *Eco*RI-*Bam*HI fragment of strain DM4-2cr. It was verified that this fragment did not hybridize with the cryptic plasmids of strain DM4-2cr and that it did not contain a *Hin*dIII restriction site. The 1.4-kb *Eco*RI-*Bam*HI fragment was inserted into plasmid pME3048 to yield plasmid pME1562. Cloning of the 2.8-kb *Bam*HI-*Pst*I *dcm* region as a *Bam*HI-*Hin*dIII fragment into pME1562 resulted in plasmid pME1563, and cloning of a *Hin*dIII-*Bam*HI fragment covering nucleotides 1280 to 2790 of the *dcm* region into pME1562 yielded plasmid pME1564.

Construction of *dcmR'-'lacZ* fusions was based on plasmid pME1568A (Table 1). Three different restriction fragments representing the 430-bp *Bgl*II-*Nar*I, the 635-bp *Bgl*II-*Eco*R47III, and the 765-bp *Bgl*II-*Nsi*I segments of the *dcm* region were cloned as *Eco*RI-*Bgl*II fragments into *Eco*RI-

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference[b]
Strains		
E. coli		
RR28	*pheS12* F⁻ *thi leu pro lac gal ara mtl xyl supE44 endA* r m *thyA⁺ recA*	12
S17-1	*thi pro hsdR hsdM⁺ recA*, chromosomally integrated RP4-2 (Tc::Mu, Km::Tn7)	27
MC1061	Δ(*lacIPOZYA*)*X74 galU galK hsdR* Δ(*ara leu*) Sm^r	5
Methylobacterium sp.		
DM4	DCM⁺	9
DM4-2cr	DCM⁻ Sm^r, derived from DM4	10
DM4-2cr(pME1523)	*dcmA⁺ dcmR⁺* Sm^r Tc^r Km^r	19
DM4-2cr(pME1541)	*dcmA⁺ dcmR⁺* Sm^r Km^r	19
DM4-2cr-63	*dcmA⁺ dcmR⁺* Km^r, chromosomally integrated plasmid pME1563	
DM4-2cr-64	*dcmA⁺* Km^r, chromosomally integrated plasmid pME1564	
Plasmids		
pUC18	Amp^r, pBR322-derived expression vector	29
pBLS, Bluescript KS(+)	Amp^r, pBR322-derived expression vector	Stratagene, San Diego, Calif.
pGEM-7Zf(+)	Amp^r, pBR322-derived expression vector	Promega Biotec, Madison, Wis.
pVK100	Km^r, Tc^r, mobilizable cloning vector derived from RK2	17
pRZ102	ColE1 plasmid with a Tn5 insertion in the colicin (*cea*) structural gene	15
pME3048	Km^r, mobilizable ColE1 replicon derived from pRZ102	30
pNM480	Amp^r, pBR322-derived vector for construction of translational fusions to *lacZ* from *E. coli*	24
pME1523	*dcmA⁺ dcmR⁺*, 4.2-kb *Bam*HI fragment containing the *dcm* region in pVK100	19
pME1541	*dcmA⁺ dcmR⁺*, 2.8-kb *Bam*HI-*Pst*I fragment containing the *dcm* region in pVK100	19
pME1537	*dcmA⁺ dcmR⁺*, 2.8-kb *Bam*HI-*Pst*I fragment containing the *dcm* region in pUC18	19
pME1562	1.4-kb *Eco*RI-*Bam*HI randomly cloned chromosomal DNA fragment from strain DM4-2cr in pME3048	
pME1563	*dcmA⁺ dcmR⁺*, 2.8-kb *Bam*HI-*Pst*I fragment containing the *dcm* region in pME1562	
pME1564	*dcmA⁺*, 1.5-kb *Hind*III-*Pst*I(*Bam*HI) fragment from pME1545 (19) in pME1562	
pME1568	3.5-kb *Eco*RI-*Asu*II fragment of pNM480 in pGEM-7Zf(+)	
pME1568A	Multiple cloning site of pME1568 from *Asu*II to *Nsi*I exchanged by an *Xho*I site	

[a] DCM⁺, DCM utilizing; DCM⁻, DCM nonutilizing.
[b] From this work unless otherwise indicated.

*Bam*HI-digested pME1568A. Subcloning of the resulting constructs as *Xho*I fragments into pVK100 yielded the *dcmR'-'lacZ* fusion plasmids pME1575, pME1577, and pME1579.

Mobilization of plasmids. For expression studies, recombinant plasmids were mobilized from *E. coli* S17-1 into *Methylobacterium* strains as described elsewhere (19).

Preparation of crude extracts. *Methylobacterium* sp. was grown on minimal medium with either 30 mM methanol or 32 mM DCM as the carbon source. After cells were harvested, cell suspensions were passed twice through a French pressure cell and centrifuged to obtain crude extracts. Details of the procedure have been described previously (19).

DCM dehalogenase assay. Enzyme activity was determined at 30°C by following the rate of formaldehyde production in a colorimetric test as described previously (19, 20). Enzyme activity is expressed in millikatals, 1 mkat corresponding to the amount of activity catalyzing the conversion of 1 mmol of substrate per s.

β-Galactosidase assay. *Methylobacterium* strains were grown in 30 ml of minimal medium with methanol or DCM as the carbon source (19) to an A_{600} of 0.1 to 0.2. The cultures (100 μl) were taken for assaying β-galactosidase activity as described by Miller (23).

Nuclease S1 mapping. Cellular RNA was isolated from *Methylobacterium* strains as described previously (19). Nuclease S1 mapping experiments with various 5'-³²P-end-labeled DNA fragments were carried out as outlined by La Roche and Leisinger (19). The amount of RNA per experiment varied from 20 to 50 μg. Each experiment was performed in parallel at hybridization temperatures of 54, 60, and 66°C. For nuclease S1 digestion, the concentration of the enzyme varied from 1 to 2 U/μg of cellular RNA. The lengths of the nuclease S1-resistant hybrids were analyzed on a denaturing sequencing gel with 3'-³²P-end-labeled *Hpa*II- or *Hinf*I-digested pBR322 DNA as length standards.

Isolation of total cellular DNA and plasmid DNA from *Methylobacterium* strains. The procedures for the isolation of total cellular DNA and for the purification of plasmids from *Methylobacterium* sp. have been described previously (10). Cells were grown on methanol as the carbon source.

Nucleotide sequence accession number. The coding sequence of the 2.8-kb *Bam*HI-*Pst*I fragment is filed in the GenBank data base under accession number M32346.

RESULTS

Construction of a stable, regulation-defective *Methylobacterium* strain. We have shown previously that pVK100 derivatives carrying the 2.8-kbp *Bam*HI-*Pst*I *dcm* region restore

Bam HI Sal I Bgl II Nar I Eco 47 III Nsi I Bsp MI Nar I Eco RI Nar I Pst I

0   400   800   1200   1600   2000   2400   2790 bp

ORF B1   ORF A1   ORF C1

dcm R   dcm A

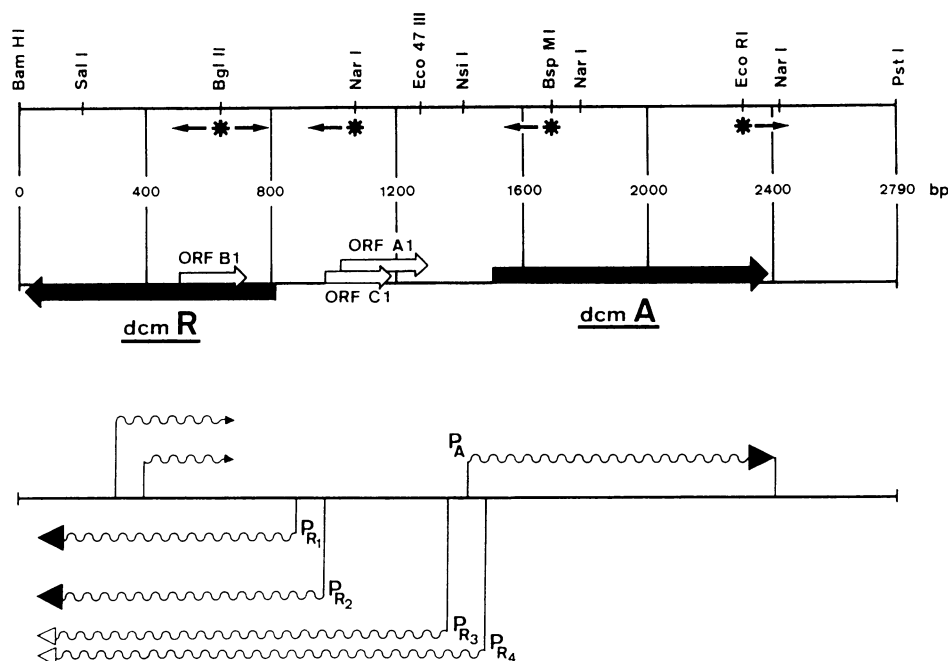P_A   P_{R_1}   P_{R_2}   P_{R_3}   P_{R_4}

FIG. 1. Restriction map, ORFs, and in vivo transcripts of the previously sequenced (19) 2.8-kb *dcm* region. Asterisks and arrows below the restriction map mark the ^{32}P-labeled end and the orientation of DNA fragments used as probes in the nuclease S1 mapping experiments (Table 2). Heavy black arrows indicate genes, and white arrows indicate ORFs. Wavy lines show mRNAs detected by nuclease S1 mapping with RNA isolated from strain DM4-2cr(pME1541). Black arrowheads indicate that the corresponding mRNA was observed with RNA from methanol- and DCM-grown cells. White arrowheads indicate signals obtained with RNA only from DCM-grown cells. Vertical lines mark the 5' ends of the corresponding mRNA and the 3' end of *dcmA* mRNA.

regulated DCM dehalogenase synthesis in the DCM-nonutilizing strain DM4-2cr (19). In this strain, a DNA fragment larger than 21 kb and spanning the *dcm* region is deleted (10). Introduction of plasmids with deletions in the *dcmA* upstream region into strain DM4-2cr led to transconjugants with constitutive expression of DCM dehalogenase. Such regulation-defective strains, however, proved unstable. The analysis of single colonies, obtained at different intervals after mobilization of the relevant constructs into strain DM4-2cr, revealed that the DCM utilization (DCM$^+$) phenotype was progressively lost. This loss occurred under nonselective conditions during growth on methanol, irrespective of whether tetracycline was applied to select for vector maintenance. The molecular events underlying the loss of the DCM$^+$ phenotype were not explored.

To overcome the instability of plasmids conferring constitutive *dcmA* expression, the 2.8-kb *dcm* region and its truncated version represented by the 1.5-kb *Eco*47III-*Pst*I fragment (Fig. 1) were integrated into the chromosome of strain DM4-2cr. The construction of the two strains with chromosomal insertions was based on the mobilizable ColE1 replicon pME3048 (30) (see Materials and Methods), which is unable to replicate in *Methylobacterium* sp. strain DM4. Insertions into the multiple cloning site of pME3048 comprised a 1.4-kb *Eco*RI-*Bam*HI fragment of randomly cloned chromosomal DNA from strain DM4-2cr to provide a target for homologous recombination and the 2.8- or 1.5-kb DNA fragments of the *dcm* region to be integrated into the chromosome. Mobilization of these constructs (plasmids pME1563 and pME1564) (Table 1) from *E. coli* S17-1 and their forced integration by selection for kanamycin resistance into the chromosome of strain DM4-2cr resulted in strains DM4-2cr-63 and DM4-2cr-64 (Fig. 2). Single-colony

analysis showed that the DCM$^+$ phenotype was stably maintained in both strains, and the chromosomal locations of the integration vectors pME1563 and pME1564 were verified by Southern analysis of genomic DNA (data not shown).

As shown in the upper part of Fig. 2, the DCM dehalogenase-specific activities of strains DM4-2cr-63 and DM4-2cr-64 grown on DCM (inducing conditions) amounted to approximately 1.4 mkat/kg of protein. This value is 3 times lower than the specific activity of 4.0 mkat/kg of protein measured in induced cells of the wild-type strain DM4 (19). Since the basal specific activity in extracts of strain DM4-2cr-63 grown on methanol (noninducing conditions) was also lowered with respect to the wild type, the induction factor for DCM dehalogenase of about 50 was unaffected in the construct carrying the chromosomally inserted *dcm* region. The lowered DCM dehalogenase levels in strains DM4-2cr-63 and DM4-2cr-64 remain unexplained. They were reflected by substantially decreased growth rates of the strains on DCM. Dehalogenase formation in strain DM4-2cr-64 was fully constitutive (Fig. 2). This confirmed the notion (19) that a regulatory factor exerting negative control over dehalogenase synthesis is encoded in the left-hand part (nucleotides 1 to 1500) of the *dcm* region.

Transcripts of the *dcm* region. The nucleotide sequence of the 2.8-kb *dcm* region has been determined previously (19). A search for open reading frames (ORFs) potentially encoding proteins larger than 7,000 Da revealed, in addition to *dcmA*, four ORFs fulfilling this criterion. The positions of these ORFs on the map of the *dcm* region are indicated in Fig. 1. Nuclease S1 mapping was used to determine whether any of these ORFs were transcribed into mRNA. Cellular RNA was isolated from strain DM4-2cr(pME1541) grown on methanol or on DCM. The 5'-^{32}P-end-labeled DNA frag-
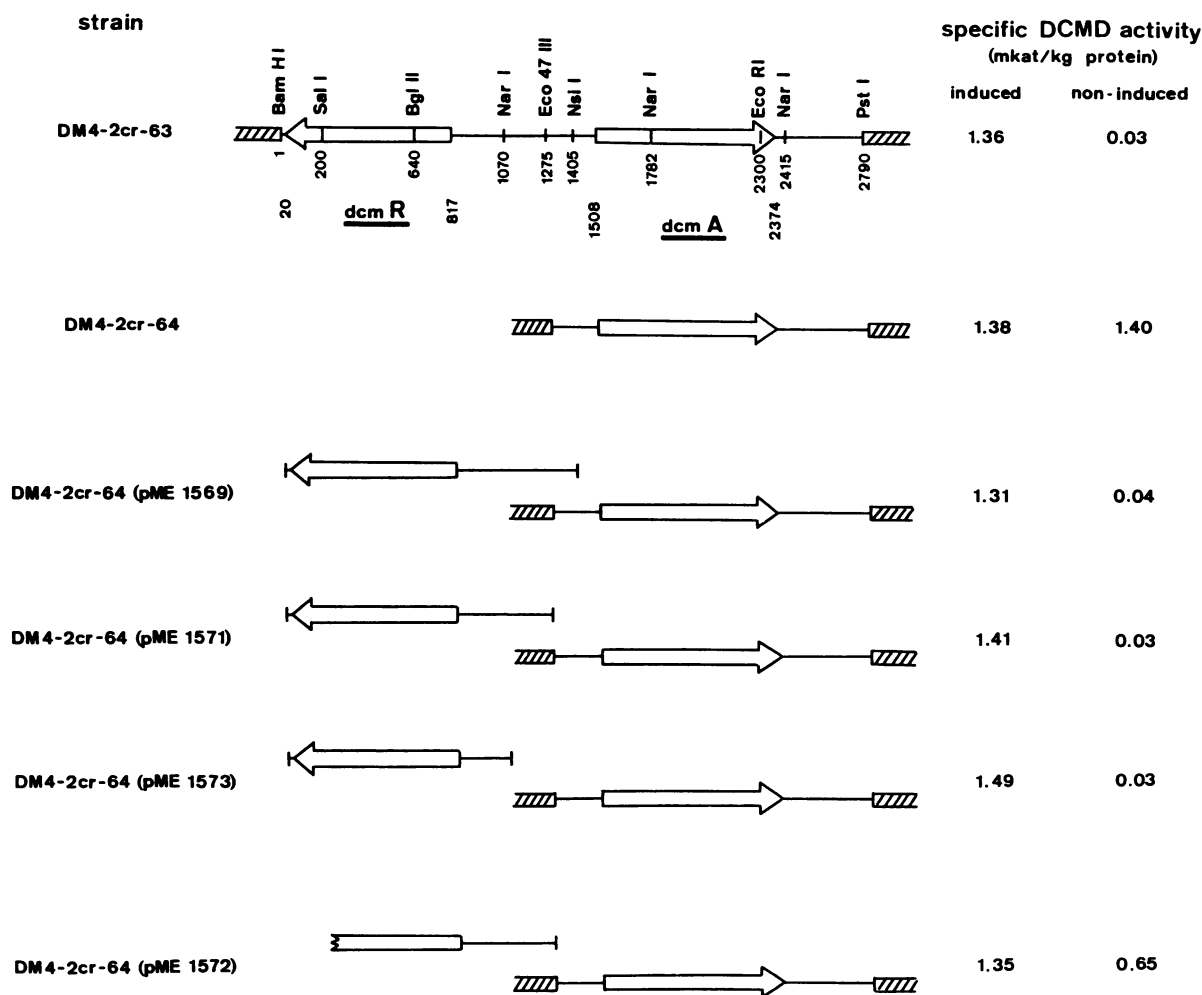
FIG. 2. Complementation of the regulation-defective mutant strain DM4-2cr-64 of *Methylobacterium* sp. by cloned fragments of the *dcmA* upstream region. The top line shows a restriction map of the chromosomally integrated *dcm* region in strain DM4-2cr-63. Nucleotide sequence positions of restriction sites as well as of the translational start and endpoints of *dcmA* and *dcmR* are indicated. Transconjugants tested for restoration of regulation carry the *dcmA* gene in the chromosome (hatched) and complementing DNA fragments on the vector pVK100. Crude extracts of methanol- and DCM-grown cells were prepared, and the specific activity of DCM dehalogenase (DCMD) was measured.

ments used to detect the possible transcripts of each ORF are listed in Table 2, and the results of the nuclease S1 mapping experiments are summarized in Fig. 1.

No nuclease S1-resistant hybrids were detected from ORF A1 and ORF C1. Two transcripts covering all or part of ORF B1 produced weak signals, which were not further analyzed. However, *dcmR*, a gene oriented in the opposite direction of *dcmA*, was transcribed into a number of RNA species (Fig. 1). The best candidates for promoters of *dcmR* are the DNA regions that give rise to mRNA species at positions 881 (P_{R1}), 975 (P_{R2}), 1360 (P_{R3}), and 1480 (P_{R4}) of the *dcm* nucleotide sequence (Fig. 3). However, the three lower-molecular-weight mRNA species could, in principle, also represent processed products. The other bands visible in Fig. 3 are less likely to originate from transcription initiation at promoters or from processing of larger transcripts, since they are represented rather strongly in the control lane with the labeled DNA probe. RNA isolated from DCM-grown cells gave rise to all four signals, whereas RNA preparations from methanol-grown cells yielded only the bands corresponding to P_{R1} and P_{R2} (Fig. 1 and 3). The relative intensity

of the signal corresponding to P_{R1} varied, depending on whether the RNA originated from DCM- or methanol-grown cells. The signal was more pronounced in RNA obtained after growth on DCM, and this observation suggested transcriptional control of *dcmR* expression.

The nuclease S1 mapping experiments also yielded information on the position of the 3' end of *dcmA* mRNA. Cellular RNA was isolated from strains DM4 and DM4-2cr(pME 1523). With the 3'-^{32}P-end-labeled fragment listed in Table 2 as a probe, two signals corresponding to mRNA 3' ends at sequence positions 2405 and 2406 were observed with RNA preparations from cells grown on methanol or DCM (Fig. 1; Table 2). *dcmA* mRNA thus terminated approximately 9 bp upstream of a 34-bp G+C-rich sequence of imperfect dyad symmetry with the potential to form a stem-loop structure ($\Delta G^0 = -43$ kcal [ca. -190 kJ/mol]) (19). This region may be specifically involved in termination of transcription.

dcmR encodes a trans-active repressor of dcmA expression. The 1.5-kb fragment of the *dcm* region necessary for regulated dehalogenase expression was further analyzed. Re-

TABLE 2. ORFs in the 2.8-kb *dcm* region and results of nuclease S1 mapping experiments

ORF or gene	Sequence positions of translation[a]		Mol wt of predicted protein	DNA fragment used for nuclease S1 mapping (sequence position)	Sequence position of S1 mapping signal (promoter)
	Start	End			
dcmR	817	20	30,110	5'-^{32}P-*Bgl*II (640) to *Bsp*MI (1690)	881[b] (P_{R1}) 975[b] (P_{R2}) 1360[c] (P_{R3}) 1480[c] (P_{R4})
ORF B1	515	724	7,740	5'-^{32}P-*Bgl*II (640) to *Bam*HI (1)	310[b] 400[b]
ORF A1	1027	1395	13,130	5'-^{32}P-*Nar*I (1070) to *Bam*HI (1)	
ORF C1	978	1184	7,350	5'-^{32}P-*Nar*I (1070) to *Bam*HI (1)	
dcmA[d] (5' end)	1508	2374	33,080	5'-^{32}P-*Bsp*MI (1690) to *Bgl*II (640)	1426[b] (P_A)
dcmA (3' end)	1508	2374	33,080	3'-^{32}P-*Eco*RI to *Pst*I (2790)	2406[b]

[a] Sequence positions are numbered relative to the *Bam*HI restriction site at position 1 of the published *dcm* nucleotide sequence (19).
[b] Signal observed with RNA isolated from cells grown on DCM or methanol.
[c] Signal observed with only RNA from cells grown on DCM.
[d] Data from reference 19 with corrected molecular weight of DCM dehalogenase.

striction fragments spanning various segments of this region were subcloned in plasmid pVK100 to yield plasmids pME1569, pME1571, pME1572, and pME1573 (Fig. 2). These constructs were mobilized into the regulation-defective recipient DM4-2cr-64 and tested for the ability to restore regulated expression of *dcmA*. As shown by the DCM dehalogenase-specific activities presented in Fig. 2, plasmid pME1569, which provided the entire *dcm* regulatory region in *trans*, fully complemented the regulation-negative phenotype of the host. The same applied to pME1571 and pME1573, two plasmids carrying progressive deletions of the *dcmR* upstream region. The *dcmR* promoters P_{R3} and P_{R4} (Fig. 1), thus, were not essential for *dcmA* regulation. However, in the experiment with plasmid pME1572, which carried a 0- to 200-bp deletion of the *dcm* region, the putative repressor encoded by *dcmR* was largely ineffective. The phenotype provided by pME1572 supported the view that *dcmR* and not ORF B1, which was not affected in this construct, represents the structural gene of the repressor protein. In conclusion, the intact *dcmR* gene plus 253 bp of its upstream region appear to be sufficient for complementation in *trans* of the regulation-defective phenotype.

Regulation of *dcmR* expression. The putative promoter regions of *dcmA* and *dcmR* each contained 12-bp sequences that matched in 11 of 12 bp. These sequences were located at the same positions (−14 to −25) relative to the mRNA start sites (+1) at promoters P_A and P_{R1} (Fig. 4). It appears likely that these largely homologous 12-bp sequences within the putative promoter regions represent at least part of the binding sites for the DcmR protein. Such an arrangement, with both the *dcmA* promoter as well as the *dcmR* promoter containing an operator site, would imply autoregulation at the transcriptional level of the *dcmR* gene product.

To test the possibility that DcmR was autoregulated, translational fusions of the *Methylobacterium* sp. *dcmR* gene to the *E. coli lacZ* gene were prepared. The three *dcmR'*-'*lacZ* fusion plasmids examined in Table 3 contained the first 177 bp of the *dcmR* gene fused to *lacZ* plus 588 (pME1575), 458 (pME1577), or 253 bp (pME1579) of the *dcmR* upstream sequence. They were mobilized into the *dcmR*⁺ strain DM4-2cr-63 and into the *dcmR* mutant strain DM4-2cr-64, and the resulting transconjugants were assayed for β-galactosidase activity (23) produced under inducing (growth on DCM) and noninducing (growth on methanol) conditions. As
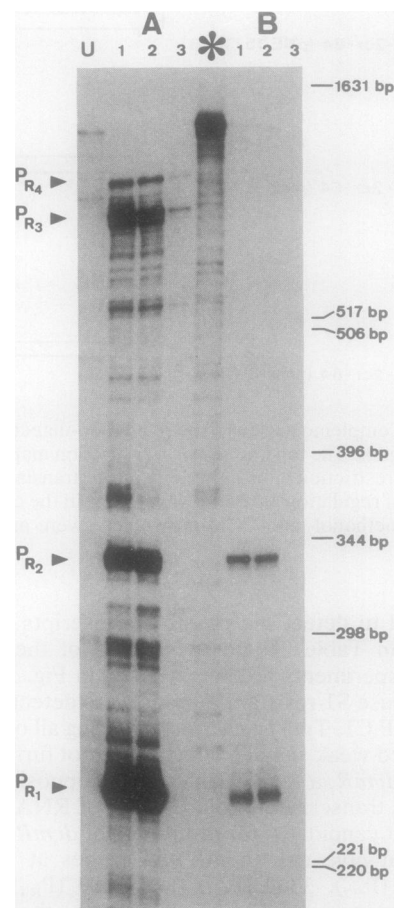

FIG. 3. Nuclease S1 mapping of *dcmR* transcripts. RNA (50 μg per experiment per lane) was taken from *Methylobacterium* sp. strain DM4-2cr(pME1523) grown on DCM (A, lanes 1 through 3) or methanol (B, lanes 1 through 3). The results of a control without RNA are shown in lane U, and the asterisk indicates the lane loaded with the untreated 5'-^{32}P-end-labeled probe, which extended from *Bsp*MI (sequence position 1690) to *Bgl*II (sequence position 640). Hybridization temperatures were 54 (lanes 1), 60 (lanes 2 and U), and 66°C (lanes 3). Lengths of the *Hin*fI-digested pBR322 DNA used as a standard (see Materials and Methods) are indicated to the right.
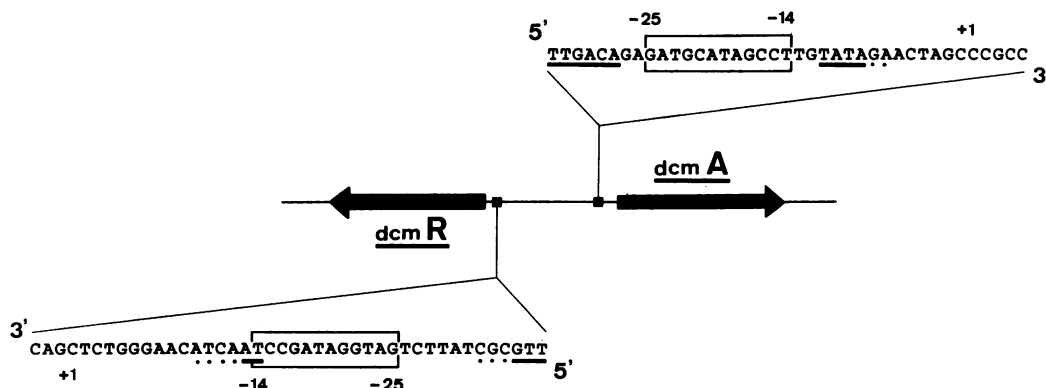
FIG. 4. Shared 12-bp sequences (boxed) in the putative promoter regions of *dcmA* and *dcmR*. The nucleotide sequences of P_A, the putative promoter of *dcmA*, and of P_{R1}, a putative promoter of *dcmR*, are shown and numbered relative to the transcriptional start sites at +1. Homologies to the *E. coli* −10 to −35 consensus promoter are underlined.

shown in Table 3, β-galactosidase-specific activity was regulated in these strains by a factor of two, and this regulation depended on the presence of a functional *dcmR* gene. The same levels of β-galactosidase were observed in the background of strain DM4-2cr-64, which did not permit DCM-dependent control, and in the background of strain DM4-2cr-63 grown under inducing conditions. The data also show that regulated *dcmR* expression was not affected by the extent of the *dcmR* upstream region contained in the constructs. They are in agreement with *dcmR* expression being subject to negative control by DcmR.

Induction by DCM of the DcmR-LacZ fusion proteins amounted to a factor of 2 and, thus, was markedly less pronounced than the induction by a factor of 20 to 30 observed for *dcmR* mRNA (Table 2; Fig. 3). To explore this discrepancy, we measured *dcmR* mRNA transcribed from the *dcmR'-'lacZ* construct pME1579. Cellular RNA was isolated from strains DM4-2cr-63(pME1579) and DM4-2cr-64(pME1579) grown on methanol and on DCM, and the RNA preparations were used for nuclease S1 mapping of the *dcmR* transcription start sites. As shown in Fig. 5, two nuclease S1-resistant hybrids were observed. Their lengths corresponded to the transcription start sites at promoters P_{R1} and P_{R2} (Table 2). The intensity of the signals obtained with the four different RNA preparations confirmed that only promoter P_{R1} was transcriptionally regulated and that this regulation was due to negative control by *dcmR*. Densitometer scanning of the S1 signals indicated that transcriptional

autoregulation of *dcmR* expression amounted to a factor of 10. Induction governed by DcmR thus was more pronounced at the level of mRNA production (10-fold) than at the protein level (twofold). This may be a consequence of the artifactual situation imposed by the *dcmR'-'lacZ* fusions or a reflection of some sort of posttranscriptional control.

DISCUSSION

In this work, we have identified the structural gene, *dcmR*, of a *trans*-acting factor involved in negative control at the transcriptional level of DCM dehalogenase formation. The simplest model of the *dcm* regulatory system assumes that *dcmR* encodes a repressor which, in the absence of DCM, binds to a recognition site in the promoter region of *dcmA*,
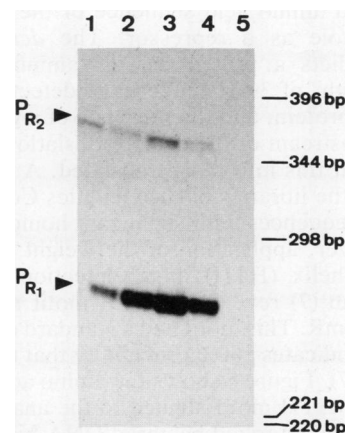
TABLE 3. β-Galactosidase activity in *dcmR⁺* and *dcmR* mutant *Methylobacterium* strains harboring *dcmR'-'lacZ* fusion constructs

Methylobacterium strain and plasmid	Presence of *dcmR*	β-Galactosidase activity (Miller units)	
		Noninduced[a]	Induced[b]
DM4-2cr-63(pME1575)	+	800	1,900
DM4-2cr-64(pME1575)	−	1,800	1,900
DM4-2cr-63(pME1577)	+	850	1,600
DM4-2cr-64(pME1577)	−	1,500	1,600
DM4-2cr-63(pME1579)	+	950	1,800
DM4-2cr-64(pME1579)	−	1,800	1,900

[a] Cells grown on methanol.
[b] Cells grown on DCM.

FIG. 5. Nuclease S1 mapping of *dcmR'-'lacZ* fusion transcripts. RNA (50 μg per experiment and lane) was taken from *Methylobacterium* sp. strain DM4-2cr-63(pME1579) (*dcmR⁺ dcmR'-'lacZ*) grown on methanol (lane 1) or DCM (lane 2), strain DM4-2cr-64 (pME1579) (*dcmR dcmR'-'lacZ*) grown on methanol (lane 3) or DCM (lane 4), and strain DM4-2cr(pME1541) (*dcmR⁺*) grown on DCM (lane 5). The positions of *Hin*fI-digested pBR322 DNA fragments used as size standards are indicated to the right. The ³²P-end-labeled DNA fragment used as a probe extended from the *Nar*I site (sequence position 1070) to the *Bgl*II site (sequence position 640) and for 10 bp into *lacZ'*. It detected exclusively *dcmR'-'lacZ* fusion transcripts and did not produce signals with *dcmR* mRNA (for comparison, see lane 5).

Helix   Turn   Helix

```
DcmR  1  MTKERSRAAKRSEEELLDIKQAASLLNVSEASLRRWTDSGKLPCYRVGDQ

     DeoR  22    LHLKDAAALLGVSEMTIRRDLN    43

     LysR  19    GSLTEAAHLLHTSQPTVSRELA    40

     NtrC 450    GHKQEAARLLGWGRNTLTRKLK   471
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 51  RARRFRREDLVAFVSVSQETASPAQGADRPDSVKNAAKTQVAGMDIAYHD

101  HICAIYGRPAGRLKLSVPLLREGLKAGDICFLNATQPGKAHILKVLREVY

151  PDVHKAIKDGQLIFPALKRNKAEMLDQLEEMFLKATYSGEQKLRLVGDME

201  WALSVDWSEQEVYEYELEYNNTLGHRFPIISLCQYDVRVFSSKGILDALK

251  SHEDTYKYPLRDCCI
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FIG. 6. Amino acid sequence of the putative DcmR repressor protein. The HTH motif of DcmR (amino acids 17 to 38) is aligned with the HTH motifs of DeoR from *E. coli* (6), LysR from *E. coli* (11), and NtrC from *Rhizobium meliloti* (28). Numbers indicate the positions of the first and the last amino acid of a HTH motif. Identical amino acids are boldfaced, and similar amino acids (Ile, Val, Leu, Met/Lys, His, Arg/Asp, Glu, Asn, Gln/Phe, Trp, Tyr/Ser, and Thr) are underlined.

the structural gene of DCM dehalogenase, and sterically hinders initiation of transcription. DCM would abolish repressor binding and thereby relieve inhibition of transcription initiation at the *dcmA* promoter. Our data are compatible with this model. However, since several important characteristics of the system remain unexplored, other more-complex regulatory circuits can, at present, not be excluded. It is not known whether DcmR, the putative repressor protein, specifically binds to the *dcmA* promoter region, and the identity of DCM as the true inducer remains to be proven. These and other points need to be clarified to validate the model presented above.

The deduced amino acid sequence of the DcmR protein supports its role as a repressor. The *dcmR* nucleotide sequence predicts a protein of 265 amino acids with a molecular weight of 30,110. We have detected the mRNA encoding this protein, and the presence of a good ribosome-binding site upstream of the dcmR translational start codon (19) argues that this mRNA is translated. A protein homology search in the libraries of the Genetics Computer Group produced no sequences with significant homology to that of DcmR. However, application of the weight matrix method for helix-turn-helix (HTH) motif detection developed by Dodd and Egan (7) revealed a HTH motif near the amino terminus of DcmR. This motif had a standard deviation score of 4.5, which indicates 100% probability that the sequence is a HTH motif (7). Figure 6 shows the amino acid sequence of DcmR with its HTH motif aligned to the analogous regions of three well-characterized bacterial DNA-binding proteins. The N-terminal location of the DcmR HTH region is a feature observed among the majority of bacterial regulatory proteins with the HTH motif (7). We found that a DcmR protein deleted for 22% of its C-terminal amino acid sequence was still marginally functional (Fig. 2). This is compatible with the N-terminal domain of DcmR being involved in DNA binding.

The two genes of the *dcm* system are organized in two separate, divergent transcription units. Several examples of divergent transcription of a structural gene and the corresponding regulatory gene exerting transcriptional control have been encountered in bacteria (4). The unusual feature

of the system presented here relates to the relatively large distance of 619 bp between the translational start sites of *dcmA* and *dcmR*. The major transcriptional start sites, P_A for *dcmA* and P_{R1} plus P_{R2} for *dcmR*, were oriented back to back, 451 bp apart (Fig. 1). Deletion of the central part of this spacing region (bp 1070 to 1275 of the *dcm* nucleotide sequence) had no effect on regulation (Fig. 2). It thus appears that the weak promoters P_{R3} and P_{R4} of *dcmR* (Fig. 1) are not important for regulation and that the spacing region encodes neither undetected regulatory sites nor structural genes. Since the two transcription units, when located on separate replicons, cooperated to yield fully regulated expression of *dcmA* (Fig. 2), their divergent orientations seem unrelated to the topology of the regulation process, for example by DNA looping (1).

The levels of DNA-binding regulatory proteins in gram-negative bacteria are frequently autogenously regulated (11, 16). We have observed *dcmR*-dependent induction of *dcmR* mRNA by DCM. Autoregulation of *dcmR* expression was pronounced at the level of transcription and much weaker at the protein level (Fig. 5; Table 3). At present, it is not clear whether this discrepancy is due to inefficient translation of the *'lacZ* reporter gene from *E. coli* (50 mol% GC) in *Methylobacterium* sp. (estimated 70 mol% GC) (13) or whether it reflects a superimposed regulatory mechanism. The latter, if true, might be based on differential stability of *dcmR* mRNA under inducing and noninducing conditions, on a mechanism involving premature termination of *dcmR* mRNA transcription, or on translational control involving DcmR (22).

The nuclease S1 mapping data (Fig. 3 and 5) and our earlier results (19) show that transcription from both promoters P_A and P_{R1} is inhibited under noninducing conditions. Both promoters contained almost identical 12-bp sequences (5'-GATG[G/C]ATAGCCT-3') covering the −14 to −25 regions. We speculate that these 12-bp segments are involved in the specific binding of the DcmR repressor. However, unlike other bacterial operator regions, they did not exhibit significant dyad symmetry within themselves or with flanking regions. If the 12-mers play a role in repressor recognition, one would expect a monomeric structure of the active DcmR protein.

In *Methylobacterium* sp. stain DM4, induction of DCM dehalogenase by its substrate is based on negative control. This seems to be an unusual case among hydrocarbon degradative pathways in gram-negative bacteria. Enzyme synthesis in the catabolism of toluene-xylene (3), naphthalene and salicylate (25), and *n*-alkanes (8) is governed by activator proteins, and another bacterial haloalkane dehalogenase, whose sequence has been established (14), is expressed constitutively. Another rare feature of the *dcm* system, negative autoregulation of repressor synthesis in a catabolic pathway, has been observed in the histidine utilization (*hut*) system of *Klebsiella aerogenes* (21) and is suspected to occur in the *hut* system of *Pseudomonas putida* (2). Regulation of histidine catabolism thus resembles, in this respect, the regulation we have observed in DCM utilization. In both systems, autoregulation of repressor synthesis would ensure high levels of repressor protein under inducing conditions and thereby prepare the cell for immediate shutdown of enzyme synthesis when the supply of DCM or histidine is depleted. In view of the high intracellular concentration of DCM dehalogenase in induced *Methylobacterium* cells (16% of the total protein) (18), such a mechanism seems particularly advantageous.

REFERENCES

1. **Adhya, S.** 1989. Multipartite genetic control elements: communication by DNA loop. Annu. Rev. Genet. **23:**227–250.
2. **Allison, S. L., and A. T. Phillips.** 1990. Nucleotide sequence of the gene encoding the repressor for the histidine utilization genes of *Pseudomonas putida*. J. Bacteriol. **172:**5470–5476.
3. **Assinder, S. J., and P. A. Williams.** 1990. The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv. Microbiol. Physiol. **31:**1–69.
4. **Beck, C. F., and R. A. J. Warren.** 1988. Divergent promoters, a common form of gene organization. Microbiol. Rev. **52:**318–326.
5. **Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou.** 1983. β-Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. **100:**293–308.
6. **Dodd, I. B., and J. B. Egan.** 1987. Systematic method for the detection of potential λ Cro-like DNA-binding regions in proteins. J. Mol. Biol. **194:**557–564.
7. **Dodd, I. B., and J. B. Egan.** 1990. Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. Nucleic Acids Res. **18:**5019–5026.
8. **Eggink, G., H. Engel, W. G. Meijer, J. Otten, J. Kingma, and B. Witholt.** 1988. Alkane utilization in *Pseudomonas oleovorans*. J. Biol. Chem. **263:**13400–13405.
9. **Gälli, R., and T. Leisinger.** 1985. Specialized bacterial strains for the removal of dichloromethane from industrial waste. Conserv. Recycl. **8:**91–100.
10. **Gälli, R., and T. Leisinger.** 1988. Plasmid analysis and cloning of the dichloromethane utilization genes of *Methylobacterium* sp. DM4. J. Gen. Microbiol. **134:**943–952.
11. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. USA **85:**6602–6606.
12. **Hennecke, H., J. Günther, and F. Binder.** 1982. A novel cloning vector for the direct selection of recombinant DNA in *E. coli*. Gene **19:**231–234.
13. **Hood, D. W., C. S. Dow, and P. N. Green.** 1987. DNA:DNA hybridization studies on the pink-pigmented facultative methylotrophs. J. Gen. Microbiol. **133:**709–720.
14. **Janssen, D. B., F. Pries, J. van der Ploeg, B. Kazemier, P. Terpstra, and B. Witholt.** 1989. Cloning of 1,2-dichloroethane degradation genes of *Xanthobacter autotrophicus* GJ10 and expression and sequencing of the *dhlA* gene. J. Bacteriol. **171:**6791–6799.
15. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177:**65–72.
16. **Kelley, R. L., and C. Yanofsky.** 1982. *trp* aporepressor production is controlled by autogeneous regulation and inefficient translation. Proc. Natl. Acad. Sci. USA **79:**3120–3124.
17. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8:**45–54.
18. **Kohler-Staub, D., S. Hartmans, R. Gälli, F. Suter, and T. Leisinger.** 1986. Evidence for identical dichloromethane dehalogenases in different methylotrophic bacteria. J. Gen. Microbiol. **132:**2837–2843.
19. **La Roche, S. D., and T. Leisinger.** 1990. Sequence analysis and expression of the bacterial dichloromethane dehalogenase structural gene, a member of the glutathione S-transferase supergene family. J. Bacteriol. **172:**164–171.
20. **Leisinger, T., and D. Kohler-Staub.** 1990. Dichloromethane dehalogenase from *Hyphomicrobium DM2*. Methods Enzymol. **188:**355–361.
21. **Magasanik, B., and F. C. Neidhardt.** 1987. Regulation of carbon and nitrogen utilization, p. 1318–1325. *In* F. C. Neidhardt, J. L. Ingraham, K. Brooks Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
22. **McCarthy, J. E. G., and C. Gualerzi.** 1990. Translational control of prokaryotic gene expression. Trends Genet. **6:**78–85.
23. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Minton, N. P.** 1984. Improved plasmid vectors for the isolation of transcriptional *lac* gene fusions. Gene **31:**269–273.
25. **Schell, M. A., and E. F. Poser.** 1989. Demonstration, characterization, and mutational analysis of NahR protein binding to *nah* and *sal* promoters. J. Bacteriol. **171:**837–846.
26. **Scholtz, R., L. P. Wackett, C. Egli, A. M. Cook, and T. Leisinger.** 1988. Dichloromethane dehalogenase with improved catalytic activity isolated from a fast-growing dichloromethane-utilizing bacterium. J. Bacteriol. **170:**5698–5704.
27. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–790.
28. **Szeto, W. W., B. T. Nixon, C. W. Ronson, and F. M. Ausubel.** 1987. Identification and characterization of the *Rhizobium meliloti ntrC* gene: *R. meliloti* has separate regulatory pathways for activation of nitrogen fixation genes in free-living and symbiotic cells. J. Bacteriol. **169:**1423–1432.
29. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
30. **Voisard, C.** 1988. Etude génétique de *Pseudomonas fluorescens* CHAO, une souche antagoniste de champignons phytopathogènes. Thesis nr. 8663. Swiss Federal Institute of Technology, Zürich, Switzerland.