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Gene *mdpC* Plays a Regulatory Role in the Methyl-*tert*-butyl Ether Degradation Pathway of *Methylibium petroleiphilum* Strain PM1

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Gene *mdpC* plays a regulatory role in the methyl-*tert*-butyl ether degradation pathway of *Methylibium petroleiphilum* strain PM1

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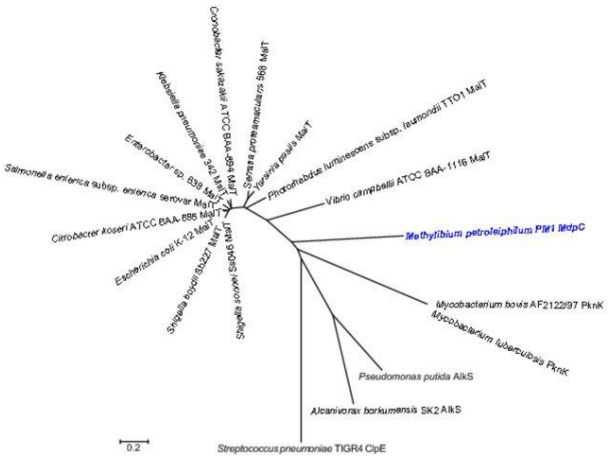
Abstract: Among the few bacteria known to utilize methyl *tert*-butyl ether (MTBE) as a sole carbon source, *Methylibium petroleiphilum* PM1 is a well-characterized organism with a sequenced genome; however, knowledge of the genetic regulation of its MTBE degradation pathway is limited. We investigated the role of a putative transcriptional activator gene, *mdpC*, in the induction of MTBE-degradation genes *mdpA* (encoding MTBE monooxygenase) and *mdpJ* (encoding *tert*-butyl alcohol hydroxylase) of strain PM1 in a gene-knockout mutant *mdpC*⁻. We also utilized quantitative reverse transcriptase PCR assays targeting genes *mdpA*, *mdpJ* and *mdpC* to determine the effects of the mutation on transcription of these genes. Our results indicate that gene *mdpC* is involved in the induction of both *mdpA* and *mdpJ* in response to MTBE and *tert*-butyl alcohol (TBA) exposure in PM1. An additional independent mechanism may be involved in the induction of *mdpJ* in the presence of TBA.

Keywords: MTBE, *Methylibium petroleiphilum* strain PM1, MdpC, regulation, degradation pathway, *mdpC*⁻ mutant

Abstract



Graphical Abstract Figure. This study utilizes mutant analysis to underline the importance of a regulatory gene in a bacterial pathway involved in the biodegradation of methyl-*tert*-butyl ether, a groundwater pollutant in the United States.

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Introduction

Methyl-*tert*-butyl ether (MTBE) was used as a fuel oxygenate in the United States and Europe for almost two decades. In California, it was banned from use in 2003 but has left a legacy of groundwater contamination in all parts of the state (Richardson and Ternes 2005) and throughout the United States (Hatzinger *et al.* 2001). One of the biodegradation products of MTBE is tertiary butyl alcohol (TBA), a potential carcinogen.

Several bacterial species and strains can mineralize MTBE and TBA including *Methylibium petroleiphilum* PM1 (Hanson, Ackerman and Scow 1999; Nakatsu *et al.* 2006), *Methylibium* sp. R8 (Rosell *et al.* 2007), *Hydrogenophaga flava* ENV735 (Hatzinger *et al.* 2001), *Mycobacterium austroafricanum* IFP2012 and IFP2015 (Francois *et al.* 2002, 2003; Lopes Ferreira *et al.* 2006), *Variovorax paradoxus* CL-8 (Zaitsev, Uotila and Häggblom 2007) and *Aquincola tertiaricarbonis* L108 (Lechner *et al.* 2007).

The involvement and efficacy of strain PM1 in aerobic biodegradation of MTBE has been demonstrated in several field studies (Wilson, Mackay and Scow 2002; Smith *et al.* 2005; Hicks *et al.* 2014). PM1-like bacteria have also been found in several MTBE-contaminated groundwater aquifers in California and play a significant role in the biodegradation process (Kane *et al.* 2001; Hristova *et al.* 2003; North *et al.* 2012a,b). The MTBE-degradation pathway of strain PM1 consists of genes present on an extrachromosomal megaplasmid that are likely controlled as a regulon; genes *mdpA* and *mdpJ* have been proposed to play a role in the degradation of MTBE and TBA, respectively (Hristova *et al.* 2007). Mutagenesis studies indicate that the gene *mdpA*, encoding MTBE monoxygenase, is involved in the first step of the degradation pathway, converting MTBE into hydroxymethyl-*tert*-butyl ether (Schmidt *et al.* 2008). Real-time qPCR assays targeting genes *mdpA* and *mdpJ* (encoding TBA hydroxylase) indicated the induction of transcription of these genes in response to MTBE and TBA (G. Joshi, unpublished data).

A putative ATP-dependent transcriptional regulator has been identified within the MTBE-degradation gene cluster, designated Mpe_B0601 or *mdpC* (Kane *et al.* 2007). To examine the role of *mdpC*

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as a regulator of the MTBE-degradation pathway of strain PM1, we developed an *mdpC* insertion-mutant strain of PM1 and designed an RT-qPCR assay to quantify *mdpC* transcription in the presence or absence of MTBE and TBA.

Materials and Methods

Culture growth and preparation

Methylibium petroleiphilum PM1 cultures were routinely grown in 0.33X tryptic soy broth (TSB) at 28°C with rotary shaking at 150 rpm or on 0.33X TSB agar at 28°C. When required, antibiotics were used in the following final concentrations: kanamycin (Km), 50 μg mL^{-1}; streptomycin (Sm), 50 μg mL^{-1}; spectinomycin (Spm) 50 μg mL^{-1}; ampicillin (Ap) 100 μg mL^{-1}. For experiments examining gene transcription, cultures were grown in mineral salts medium (MSM) (Schmidt *et al.* 2008) supplemented with 500 mg L^{-1} pyruvate and harvested at mid-log phase (OD_{595nm} = 0.25 – 0.4). *Escherichia coli* DH5α cells (Life Technologies, Grand Island, NY, USA) were used for all transformations that involved vectors carrying Sm resistance. For all other transformations, *E. coli* TOP10 (Life Technologies) cells were used. All *E. coli* cultures were grown on Luria–Bertani (LB) agar at 37°C.

Construction of mdpC *knockout strain of* M. petroleiphilum *PM1*

A primer set (mdpC-F 5′-GCAGGTGAGCAACAACCTCT-3′ and mdpC-R 5′-GACTTGTCCCGACTGCTCAT-3′) was designed to amplify *mdpC* from PM1 genomic DNA. The PCR product was cloned into pCR-XL-TOPO (Life Technologies,) using the manufacturer's protocol. Briefly, clones were screened by PCR using kit-supplied primers, confirmed by sequencing, and subsequently used for *in vitro* mutagenesis. Equimolar amounts of a correct clone (pGJ001) and EZ-Tn5<SmQ> (Schmidt *et al.* 2008) were mixed together with transposase and incubated to disrupt *mdpC*. The reaction was stopped and DNA was transformed into *E. coli* DH5α cells. Transformants were selected on LB agar containing 50 μg mL^{-1} Km and Sm each. Transposon inserts were screened by PCR using *mdpC*-specific

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primers. The exact site of insertion of likely candidates was determined by sequencing. Selected plasmid (pGJ001-SmQ) was extracted and the *mdpC*-SmQ fragment amplified using primers supplied by manufacturer. The purified amplicon (0.6 pmol; Qiagen PCR purification kit; Qiagen) was transformed into PM1 cells washed in 10% glycerol by electroporation in MicroPulser Electroporator (Bio-Rad Laboratories, Hercules, CA, USA) at 1.8 kV for 5 ms. Transformants were selected on 0.33X TSB agar containing 50 μg mL^{-1} Sm and Spm each. Potential mutants were screened by PCR and confirmed by sequencing of the PCR product.

Resting-cell experiments

One liter of pyruvate grown PM1 cultures were harvested by centrifugation at 6000 rpm for 10 min, washed twice in MSM with no carbon source and finally resuspended in 10 mL MSM supplemented with 50 mg L^{-1} MTBE, or TBA providing an equivalent amount of carbon. Experiments were conducted in 50 mL sterile glass bottles fitted with Teflon-lined mininert valve caps (Restek Corporation, Bellefonte, PA, USA). Microcosm bottles with MSM and appropriate amounts of carbon source were incubated in dark at 28°C, 150 rpm for 3 h prior to addition of washed PM1 cells in order to facilitate equilibration of aqueous partitioning of organic compounds. Final volume in microcosm bottles was 50 mL and OD_{595nm} was 0.9–1.0. Microcosms were incubated for up to 48 h. Samples for RNA extraction (1 mL) and for analysis by gas chromatography and total protein analysis were collected aseptically using Micro-Mate Glass Syringes fitted with 20G, 6-inch stainless steel deflected point septum penetration needles with luer hub (Cadence, Inc., Staunton, VA, USA). Samples for RNA extraction were preserved with RNAProtect Bacteria Reagent (Qiagen) according to manufacturer's instructions and stored at −70°C no longer than 2 weeks prior to extraction. Samples for gas chromatography were stored in 10-mL headspace vials preserved with sodium phosphate tribasic dodecahydrate at a concentration of 1% by weight and sealed with 20-mm Teflon-lined septa and aluminum crimp caps. Samples were stored no longer than a week at 4°C prior to analysis. Abiotic controls for each microcosm were also set up, with no PM1 cells added and were sampled for analysis by GC.

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RNA extraction, reverse transcription and real-time quantitative PCR

RNA was extracted from preserved cell pellets using the RNeasy Mini Kit (Qiagen), and DNA was removed using the Ambion TURBO DNA-*free* Kit (Life Technologies). RNA was quantified using the Qubit RNA Assay Kit (Life Technologies) and converted to single-stranded cDNA using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies). cDNA (8 ng per reaction) was used as template for the qPCR assays designed to detect PM1 *mdpA*, *mdpJ* and *mdpC* transcripts. In addition, eight housekeeping genes were also quantified to serve as internal standards using the geNorm approach (Vandesompele *et al.* 2002). Primers were designed using Primer3 (Koressaar and Remm 2007; Untergrasser *et al.* 2012) and are described in Table Table1.1. Briefly, 0.5 mM of each primer was used in 25 µL PCR reactions prepared with SYBR GreenER™ qPCR SuperMix for ABI PRISM (Life Technologies, Grand Island, NY, USA) in MicroAmp optical 96-well reaction plates and run on a 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA).

Table 1. RT-qPCR assays and primers used in this study. All primers are specific to genes in *M. petroleiphilum* PM1.

Primer name	Targeted gene	Function in assay	Sequence (5′ – 3′)	PCR conditions			
				Initial denaturation	Denaturation	Annealing	Cycles
mdpA 211F	MTBE monooxygenase	Gene of interest	TCATCATCGGGATGTTGCTA	95°C, 10 min	95°C, 15 s	58°C, 1 min	40
mdpA 211R			ACCGTAGAAGACCAGCGAGA				
mdpJ 245F	TBA hydroxylase	Gene of interest	TCTCCAATGTCTTCGACTGC	95°C, 10 min	95°C, 15 s	58°C, 1 min	40
mdpJ 245R			GATTCGGATCCAGACTTCGT				
qmdpC F	Putative transcriptional activator	Gene of interest	AGCCCTCAATCAATCGGTAA	95°C, 10 min	95°C, 15 s	63°C, 1 min	40
qmdpC R			AAAAGAGCGATCCAAAGACG				
pykF F	Pyruvate kinase	Internal standard	GAGCTTCCAGTGCGAGTACC	95°C, 10 min	95°C, 15 s	58°C, 1 min	40
pykF R			TCGCTAGCCTTGAGGATCTG				
gyrA F	DNA gyrase subunit A	Internal standard	GACAAGAGCTGGGATTCGTC	95°C, 10 min	95°C, 15 s	58°C, 1 min	40

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Primer name	Targeted gene	Function in assay	Sequence (5′ – 3′)	PCR conditions			
				Initial denaturation	Denaturation	Annealing	Cycles
gyrA R			CGCCATCACCTCCTTGTATT				
gyrB F	DNA gyrase subunit B	Internal standard	AATACAACCCCGACAAGCTG	95°C, 10 min	95°C, 15 s	56°C, 1 min	40
gyrB R			CCGTCCTTCAGGTACTGCTC				
glyA F	Serine hydroxymethyl transferase	Internal standard	CAGCAACAGGTGGTGAAGAA	95°C, 10 min	95°C, 15 s	62°C, 1 min	40
glyA R			TCTTGTTGCAGGTCATGTGC				
rho F	RNA polymerase rho subunit	Internal standard	GGGCTGAGGTAGATGTCGTC	95°C, 10 min	95°C, 15 s	58°C, 1 min	40
rho R			CCTTGGAGATCGAGAACAGC				
gap1 F	glyceraldehyde-3-phosphate dehydrogenase	Internal standard	CAAGAAGCACGACATCCAGA	95°C, 10 min	95°C, 15 s	62°C, 1 min	40
gap1 R			TTCCTTGGTCGTGAAGAAGC				
gap2 F	- ″ -	Internal standard	TGAGTTCGGGGTAGATCAGC	95°C, 10 min	95°C, 15 s	58°C, 1 min	40
gap2 R			TGAGTTCGGGGTAGATCAGC				
gap3 F	- ″ -	Internal standard	TGAGTTCGGGGTAGATCAGC	95°C, 10 min	95°C, 15 s	58°C, 1 min	40
gap3 R			GGCAGTTGGTGGTACAGGAG				

Gas chromatography

MTBE and TBA were quantified on an Agilent 6890N gas chromatograph equipped with a flame ionization detector and an HP 7694 headspace autosampler as described previously (Schmidt *et al.* 2008). Organic compounds were separated using an Agilent HP1 capillary column (60 m by 1 µm by 0.320 µm).

Total protein analysis

Total protein content was determined using 1 mL samples collected at the beginning and end of incubations. Total protein was extracted with BugBuster Protein Extraction Reagent containing

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Benzonase Nuclease (EMD Millipore, Merck KGaA, Darmstadt, Germany) and was quantified using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) according to manufacturer's protocols.

Data analysis

Bacterial gene expression data were normalized by dividing gene quantity values determined by RT-qPCR with the geometric mean of gene quantities of the two (or three) most stable housekeeping genes as determined by the geNorm package for Microsoft Excel (Vandesompele *et al.* 2002). The output from the gas chromatograph was analyzed using ChemStation revision A.10.02 software (Agilent, Santa Clara, CA, USA).

Sequence analysis and generation of phylogenetic trees

Functional homologs of translated *M. petroleiphilum* PM1 coding sequence were identified using BLASTP searches against the Swiss-prot database. Multiple alignments were performed using ClustalW. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987) with bootstrap analysis (*N* = 2000) (Felsenstein 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) in MEGA5 (Tamura *et al.* 2011).

Results and Discussion

MdpC protein

MdpC is a 901 amino-acid long, predicted ATP-dependent transcriptional activator in the MalT family. Two conserved domains were identified: an AAA_16 ATPase domain between residues 28 and 149, and a LuxR DNA-binding region between residues 839 and 891. Among the top BLAST identity matches for MdpC were protein kinase PknK of *M. bovis* AF2122/97 (26%), maltose-binding regulatory protein MalT of *E. coli* K-12 (25%), alkane-responsive regulatory protein AlkS of *Alcanivorax borkumensis* SK2 (38%) and AlkS of *Pseudomonas putida* Gpo1 (20%). These proteins belong to the MalT

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family of ATP-dependent transcriptional activators. A phylogenetic tree of the top BLAST hits (Fig. (Fig.1a)1a) shows deep branching of MdpC.

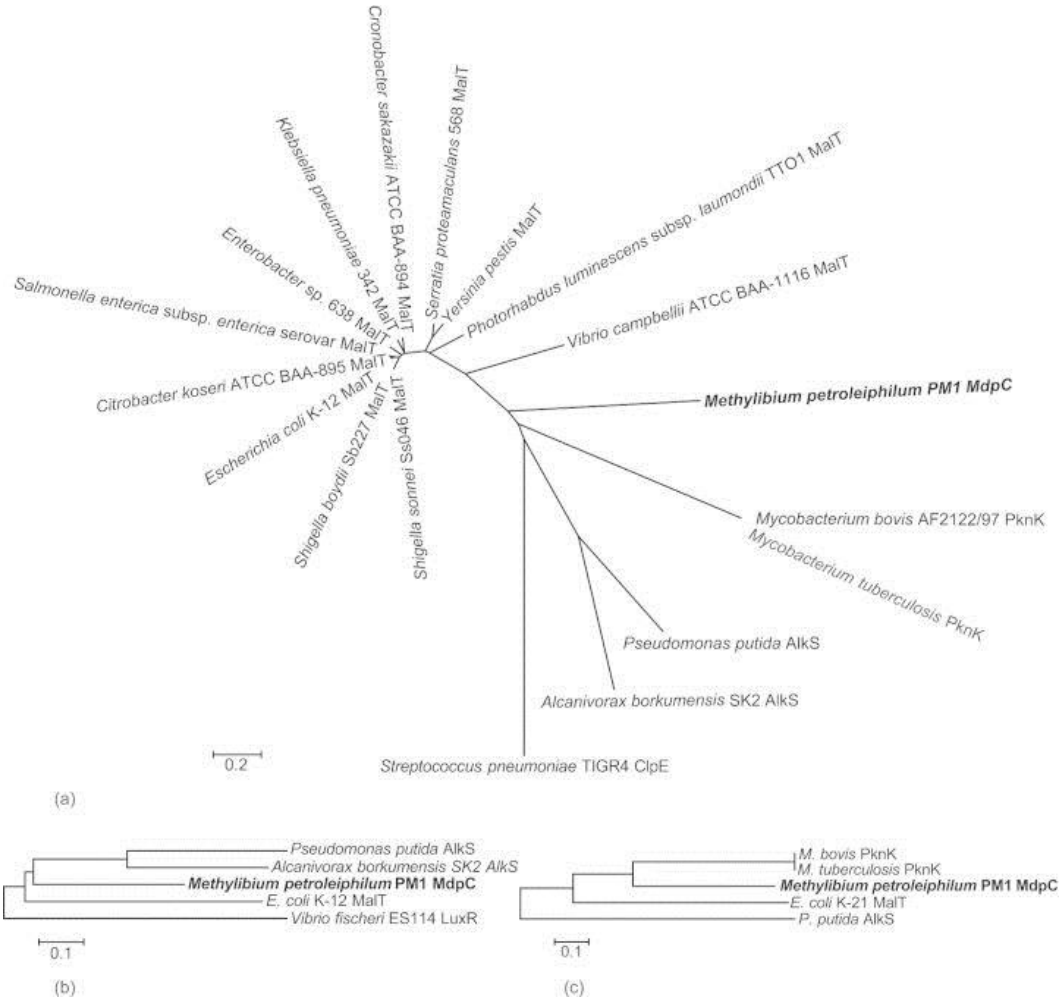

Figure 1. Phylogenetic tree of *M. petroleiphilum* strain PM1 MdpC and related ATP-dependent transcriptional activators obtained through the Swiss-prot database (**a**). The predicted transcriptional activator protein MdpC is distinct from the MalT of *E. coli* and other enteric bacteria and AlkS of *Pseudomonas* and *Alcanivorax*, and *Mycobacterium* PknK. Phylogenetic tree of *luxR* DNA-binding domains (**b**) and AAA_16 ATPase domains (**c**) of *M. petroleiphilum* MdpC and related proteins obtained through the Swiss-prot database. The predicted DNA-binding and ATPase domains of MdpC form a distinct lineage separate from related proteins. Evolutionary distances represented by branch lengths are in the units of the number of amino-acid substitutions per site.

Regions of MdpC have 23% (amino-acid positions 182–430) and 44% (amino-acid positions 838–899) identity with corresponding regions (positions 187–421 and 811–872, respectively) of AlkS from *A. borkumensis* SK2 (Hara *et al.* 2004; Reva *et al.* 2008). The AlkS of *A.*

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borkumensis SK2 has 48% similar amino-acid identity with AlkS of *P. putida*, which has been grouped within the MalT subfamily of LuxR transcriptional activators (Danot 2001; Reva *et al.* 2008). AlkS is known to be the transcriptional activator involved in the *alkB1GHJ* cluster for alkane degradation in *A. borkumensis* AP1 (van Beilen *et al.* 2004). *mdpA* of PM1 (encoding MTBE monooxygenase) is 69 and 66% identical to the alkane monooxygenase AlkB of *A. borkumensis* AP1 and *P. putida* GPo1 (Hristova *et al.* 2007). Phylogenetic trees generated from the alignments of the two predicted functional regions in MdpC with those in related proteins also show distinct branching (Fig. 1b and c).

The phosphate-binding Walker A type GYGKS motif typical of AAA_16 type ATPases at position 39–43 of MdpC is also present (as G[Y/F]GK[S/T]) in AAA_16 regions of *P. putida* AlkS (positions 54–58), *E. coli* MalT (positions 42–46) and *M. tuberculosis* PknK (positions 371–375). MpdC also has a DNA-binding, helix-turn-helix LuxR-type domain at position 839–891. Most *luxR*-type regulators act as transcription activators, but some can be repressors or have a dual role for different functional domains. The presence of this domain indicates potential role of MdpC in transcription of MTBE-degradation genes.

Effect of chloramphenicol on transcription of PM1 MTBE-degradation genes and mdpC

Chloramphenicol exposure was employed to test if inhibition of *de novo* protein synthesis affected the induction of genes *mdpA* and *mdpJ*. In the presence of MTBE, *mdpA* and *mdpJ* were induced to 7- and 42-fold of their initial levels, while *mdpC* was expressed at a constant level throughout the course of the incubation. In the presence of chloramphenicol, transcription of *mdpA, mdpJ* and *mdpC* stayed constant throughout the incubation, up to 12 h (Fig. 2a–c). MTBE was degraded by 84.3% (±10.5%) by untreated cells, whereas 38.4% (±20.9%) MTBE was degraded in chloramphenicol-treated PM1 cells after 24 h. There were no significant differences in the total protein content among the different treatments. In the presence of pyruvate alone, none of the genes under study were induced (data not shown).

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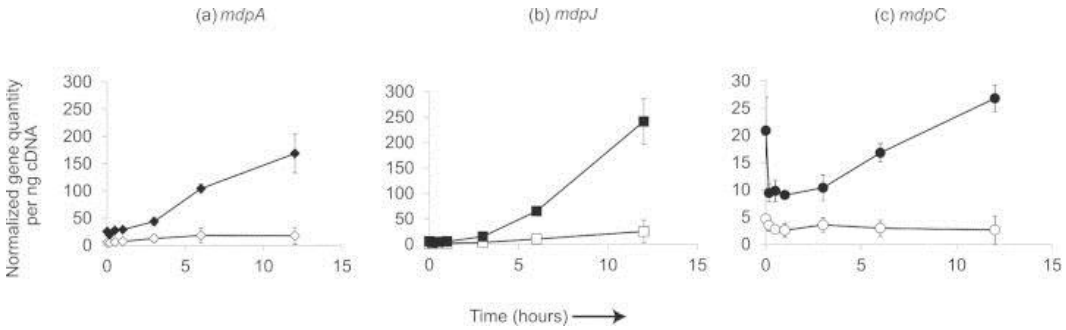

Figure 2. Effect of chloramphenicol on the transcription of PM1 MTBE-degradation genes. Induction of *mdpA* (**a**) and *mdpJ* (**b**) transcription occurred in wild-type PM1 treated with MTBE (⧫, ▪ and •) but not in cells treated with 15 μg mL^{-1} chloramphenicol (◇, □ and ◯) following exposure to 50 mg L^{-1} MTBE. There was slightly elevated transcription of *mdpC* (**c**) in the absence of chloramphenicol (MTBE alone) but was not statistically significant (P = 0.33).

Non-induction of *mdpC* in response to MTBE in strain PM1 is in contrast with AlkS of *P. putida* Gpo1, in which the mRNA associated with *alkS* increases in response to alkanes (Canosa, Yuste and Rojo 1999). In the absence of alkanes, AlkS represses P_{alkS1}. In the presence of alkanes, AlkS activates P_{alkS2}, which is a strong promoter that induces more AlkS formation, and also, P_{alkB}, which turns on a cascade of alkane-degradation genes (Canosa *et al.* 2000). Unlike AlkS, MdpC does not appear to be a self-inducer in response to the substrate MTBE.

In the presence of chloramphenicol, which is an inhibitor of prokaryotic protein synthesis, the number of transcripts of *mdpA* and *mdpJ* were reduced in wild-type PM1 cells. There were low levels of *mdpA*, *mdpJ* and *mdpC* transcripts in the presence of chloramphenicol, and these levels were similar to those measured in the presence of pyruvate (non-inducer) at time 0. A previous study has shown a low rate of MTBE degradation in PM1 cells treated with chloramphenicol (Schmidt *et al.* 2008). These results indicate that there is a low background level of mRNA of these genes in PM1 cells, and post-MTBE exposure, elevated transcription (induction) of *mdpA* and *mdpJ*.

Impact of mdpC *knockout on the transcription of MTBE-degradation genes*

There was no significant change (or induction) in the transcription of *mdpA*, *mdpJ* and *mdpC* from the initial levels after 12

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h of MTBE exposure in the mutant strain, while in the wild-type, transcription of *mdpA* was induced 13-fold and *mdpJ* was induced ~66-fold after 12 h of exposure to MTBE. A similar trend was also observed upon exposing cells to TBA (Fig. 3a–c).

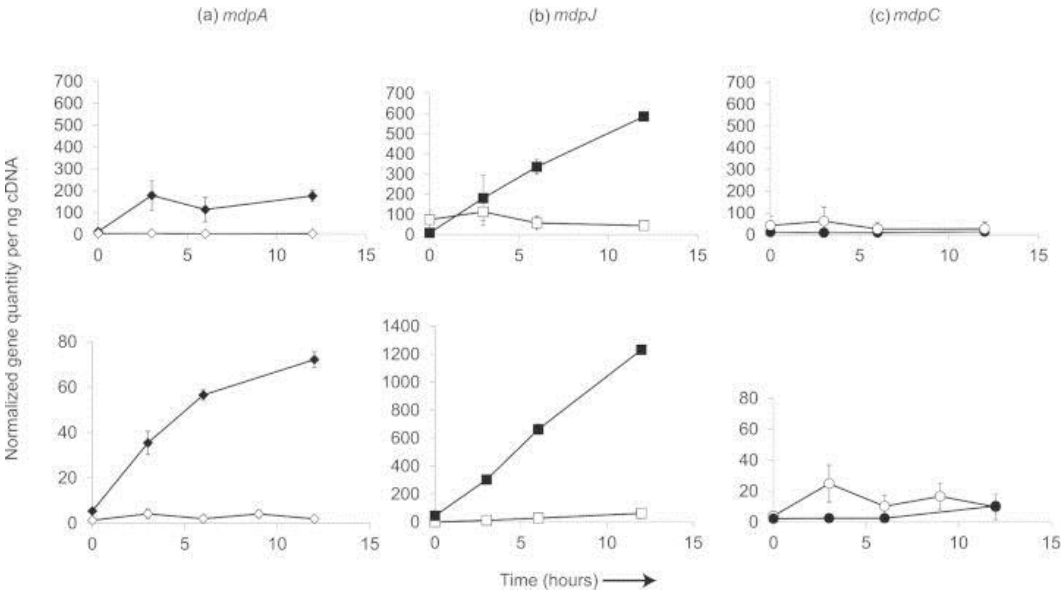

Figure 3. mdpC transcription in wild type and mdpC-knockout PM1 strains. Induction of mdpA (**a**) and mdpJ (**b**) transcription occurred in wild-type PM1 (⧫, ▪ and •) but not in the mdpC-knockout mutant (◇, □ and ◯) following exposure to 50 mg L^{-1} MTBE (top row) and 52.4 mg L^{-1} TBA (bottom row). Differences in mdpC transcription (**c**) observed in wild-type versus mutant PM1, or over 0–12h for each strain were not statistically significant ($P > 0.05$).

mdpJ transcription increased 61(±27)-fold ($P = 0.0176$) in 12 h of TBA exposure in *mdpC*⁻ cells, and 27.4 (±2.3)-fold ($P = 0.0001$) in wild-type cells. However, total number of *mdpJ* transcripts in *mdpC*⁻ cells was still 45 (±11.3)-fold lower ($P = 0.0024$) than in wild-type cells at the beginning of the incubation, and 19.9 (±0.97)-fold lower ($P = 0.0000$) after 12 h (Fig. (Fig.3b),3b), indicating that any level of induction in mutant was not as high as that in wild type. The rate of MTBE degradation was greater in wild-type cells than *mdpC*⁻ mutant after 48 h, whereas the rates of TBA degradation among the two strains were comparable (Table 2). There were no significant differences in the total protein content of the cells among the different treatments.

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Table 2. Degradation rates of carbon source by different PM1 strains. Concentrations were measured for 48 h.

	MTBE	**TBA**
Wild-type	0.036 h^{-1}	0.01 h^{-1}
mdpC⁻ mutant	0.013 h^{-1}*	0.017 h^{-1}

*Value was not statistically different from abiotic control (P = 0.52).

The induction of both *mdpA* and *mdpJ* in the presence of MTBE in the wild type but not in the mutant is suggesting a direct *mdpC* role in transcriptional activation of both genes. There is a large gap of ~54 kb between these two genes in the megaplasmid and they are not part of a single operon. The induction of *mdpJ* in mutant cells from its initial level in the presence of TBA was lower than that observed in wild type. Therefore, a second, though less efficient, regulatory mechanism independent of *mdpC* is possibly involved in the induction of *mdpJ*. Alternatively, the limited *mdpJ* induction in the presence of chloramphenicol could be due to a broad regulatory response such as cellular shock response.

The role of a regulatory protein in bacterial MTBE degradation has not been studied prior to our work. Our results provide direct evidence for the role of *mdpC* in the regulation of the MTBE-degradation pathway in strain PM1. This regulatory role is via MdpC, a transcriptional activator with MalT-like ATPase and LuxR DNA-binding domains, but overall limited homology with similar proteins in other bacteria. Future studies to address questions such as interaction of overexpressed MdpC with specific promoter regions of the MTBE-gene cluster in PM1 megaplasmid and effect of known repressors of MTBE-degradation in PM1, such as ethylbenzene (G. Joshi, unpublished data), on expression of *mdpA* and *mdpJ* in the *mdpC*⁻ mutant, will provide a detailed understanding of the mechanism of regulation of the pathway via MdpC. Additionally, it will be of interest to determine if MdpC binds directly to MTBE, or to other molecules, or a protein intermediate, in order to regulate MTBE degradation in strain PM1, through substrate binding studies with overexpressed MdpC. A deeper understanding of regulatory mechanism of the MTBE-degradation pathway of strain PM1 could aid the development of novel approaches for detection and bioremediation of these chemicals.

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Conflict of interest statement. None declared.

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