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Differential Regulation of the Three Methanol Methyltransferase Isozymes in *Methanosarcina acetivorans* C2A

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Genetic analysis of the three methanol-specific methyltransferase 1 operons (*mtaCB1*, *mtaCB2*, and *mtaCB3*) in *Methanosarcina acetivorans* led to the suggestion that each of them has a discrete function during growth on methanol, which might be reflected in differential gene regulation (Pritchett and Metcalf, Mol. Microbiol. 56:1183–1194, 2005). To test this suggestion, reporter gene fusions were constructed for each of the three operons, and their expression was examined under various growth conditions. Expression of the *mtaCB1* and *mtaCB2* fusions was 100-fold and 575-fold higher, respectively, in methanol-grown cells than in trimethylamine (TMA)-grown cells. The *mtaCB3* fusion was expressed at low levels on methanol, TMA, and dimethylamine but was significantly upregulated on monomethylamine and acetate. When TMA- or acetate-grown cultures were shifted to methanol, the *mtaCB1* fusion was expressed most highly during exponential phase, whereas the *mtaCB2* fusion, although strongly induced prior to *mtaCB1* expression, did not reach full expression levels until stationary phase. The *mtaCB3* fusion was transiently expressed prior to entry into exponential phase during a TMA-to-methanol substrate shift experiment. When acetate-grown cells were shifted to medium containing both TMA and methanol, TMA utilization commenced prior to utilization of methanol; however, these two substrates were consumed simultaneously later in growth. Under these conditions expression of the *mtaCB2* and *mtaCB3* fusions was delayed, suggesting that methylamines may repress their expression.

Methane-producing archaea (methanoarchaea) are responsible for essentially all biological methane production on earth, yet most of these organisms are metabolically limited and are able to use one or, at most, two growth substrates. Moreover, all methanoarchaea are obligate methanogens. In this group, the *Methanosarcina* species are the only organisms that possess significant metabolic versatility. Many *Methanosarcina* species can use numerous methanogenic substrates, including H_2-CO_2, carbon monoxide, acetate, methanol, trimethylamine (TMA), dimethylamine (DMA), monomethylamine (MMA), methylsulfide, and dimethylsulfide (33). This metabolic diversity is reflected in the available genome sequences of three *Methanosarcina* species, which on average are more than twice the size of other known methanoarchaeal genomes. Interestingly, multiple copies of many of the genes that are specifically required for the use of alternate methanogenic substrates are present, and the multiple copies are conserved across all three *Methanosarcina* genomes that have been sequenced. For example, two or three copies of each of the genes required for entry of one-carbon compounds (C-1 compounds), such as methanol, TMA, DMA, and MMA, into the central methanogenic pathway are present. To date, little is known regarding the evolutionary advantage of having multiple isozymes of the proteins encoded by these genes; however, recent studies have begun to address this issue with respect to methanol utilization.

C-1 compounds are disproportionated to methane and carbon dioxide in a 3:1 ratio by using the reducing equivalents generated from oxidation of one methyl group to CO_2 to reduce three additional methyl groups to methane. These C-1 compounds enter the methanogenic pathway via activation by sets of substrate-specific methyltransferases designated methyltransferase 1 (MT1) and MT2 (Fig. 1). The methanol-specific MT1 consists of two protein components present at a 1:1 ratio (24, 30): MtaC, a 24-kDa corrinoid protein, and MtaB, a 49-kDa methyltransferase that transfers the methyl group from methanol to the corrinoid prosthetic group of MtaC. Subsequently, the MT2 reaction, catalyzed by the 38-kDa MtaA protein, transfers the methyl group from the corrinoid prosthetic group of MtaC to coenzyme M (mercaptoethanesulfonic acid) (28). The *mtaC* and *mtaB* genes comprise an operon, while *mtaA* is transcribed monocistronically (10, 24).

Analysis of the *Methanosarcina acetivorans* C2A genome sequence revealed the presence of three *mtaCB* operons (*mtaCB1*, *mtaCB2*, and *mtaCB3*) and two *mtaA* genes (*mtaA1* and *mtaA2*) (9). Interestingly, these multiple gene copies are conserved in both *Methanosarcina mazei* Gö1 (5) and *Methanosarcina barkeri* strain Fusaro (GenBank accession no. NC007355). Furthermore, the multiplicity of methyltransferase-encoding genes is not limited to methanol-specific genes but is also seen for genes encoding enzymes specific for other C-1 substrates (7, 8, 26, 27). Thus, the advantages of multiple isozymes, whatever they are, may well apply to activation of all C-1 substrates.

Genetic analysis was recently used to address the role of the three *mtaCB* operons in *M. acetivorans* (21). A series of strains lacking the *mtaCB1*, *mtaCB2*, and *mtaCB3* operons in all pos-

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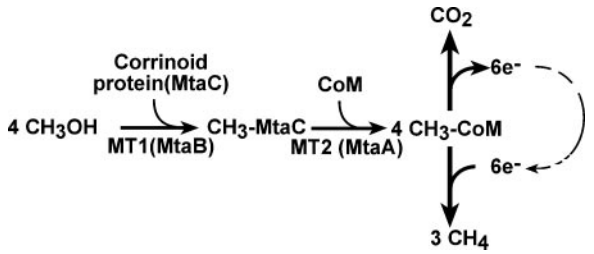

FIG. 1. Simplified scheme for methanogenesis from methanol. Methanol (CH_3OH) is converted to methyl coenzyme M (2-mercaptoethanesulfonic acid) (CoM) by the concerted effort of three proteins. Methyltransferase 1 (MT1) is comprised of methanol:5-hydroxy-benzimidazolyl-cobamide methyltransferase, MtaB, and the corrinoid-binding protein, MtaC. The product of the MT1 reaction, methyl-MtaC is the substrate for a second methyltransferase (MT2 or MtaA), which transfers the methyl group from the corrinoid protein to coenzyme M. Methyl coenzyme M (CH_3-CoM) is then disproportionated, with one molecule oxidized to CO_2 to provide the electrons required for the reduction of three CH_3-CoM molecules to methane (not all steps are shown).

sible combinations was constructed. Strains with any two of the three operons deleted were able to grow on methanol, whereas strains with all three operons deleted were not able to grow on methanol, proving that *mtaCB1*, *mtaCB2*, and *mtaCB3* all encode bona fide methanol-activating MT1 enzymes (however, this does not rule out the possibility that they can activate other substrates as well). Nevertheless, biochemical characterization of mutants showed that the three MT1 operons are not equivalent. Strains carrying only *mtaCB1* had methyltransferase activity (measured during exponential phase during growth on methanol) similar to that of the wild type, whereas the methyltransferase activities of strains carrying only *mtaCB2* or *mtaCB3* were two- and fourfold lower, respectively. Interestingly, the presence of the *mtaCB2* and *mtaCB3* operons in addition to the *mtaCB1* operon did not increase the overall methyltransferase activity. Thus, the function of the multiple gene copies cannot be simply to increase the overall methyltransferase activity. Although the growth rates and yields of most of these mutants were not affected, deletion of any one of the three operons resulted in prolonged lag phases (relative to the wild type) when the mutants were switched from other substrates to methanol. This effect was magnified in strains lacking two of the three operons. Strains carrying only *mtaCB3* were particularly affected and exhibited much slower growth, very long lag phases, and reduced cell yields on methanol medium. Taken together, these data strongly suggest that the three isozymes play discrete roles during adaptation to and growth on methanol. If this is true, it is highly likely that the *mtaCB1*, *mtaCB2*, and *mtaCB3* operons display differential gene regulation consistent with the discrete functions.

Recent data for *Methanosarcina thermophila*, *M. mazei*, and *M. acetivorans* support the idea that the MT1 isozymes are differentially regulated; however, these data are somewhat contradictory. Using two-dimensional gel electrophoresis of *M. thermophila* cell extracts coupled with mass spectrometric identification of proteins, Ding et al. showed that MtaC1, MtaB1, MtaC2, and MtaB2 were synthesized in methanol-grown cells but not in acetate-grown cells. Similar levels of MtaC3 and MtaB3 were found in extracts of both acetate- and methanol-grown cells (6). Similar results were obtained for *M. acetivorans*; MtaC1 and MtaB1 were ~15-fold more abundant in methanol-grown cells, and MtaB2 was ~33-fold more abundant in methanol-grown cells. However, in this organism the MtaC3 and MtaB3 proteins were reported to be induced approximately fivefold by growth on acetate (14, 15). These data led to the suggestion that expression of the *mtaCB3* operon prior to exposure to methanol might allow rapid adaptation from acetate to methanol. Substantially different results were obtained in a recent DNA microarray study of *M. mazei* GÖ1(11). In this study, *mtaCB1* expression was induced ~30-fold during growth on methanol compared to growth on acetate. However, the *mtaCB2* operon was not induced by methanol but was induced ~10-fold by growth on acetate. Whether these data reflect true differences between the species or whether they result from inherent limitations of the experimental methods remains to be seen.

The experimental complications involved in examining highly homologous genes led us to directly examine the regulation of the three *mtaCB* operons using reporter gene fusions to the promoters of each operon. This method, made possible by recent advances in the genetic analysis of *Methanosarcina* species, unambiguously discriminates between the three gene copies and is much simpler than other methods, and it allowed us to examine gene expression over a broader range of growth conditions. Here we describe a comprehensive analysis of *mtaCB* gene regulation in *M. acetivorans*. In addition to examining the expression of the operons during exponential growth on methanol and acetate, we examined the levels of expression on TMA, DMA, and MMA, the temporal pattern of expression throughout growth during shifts from methylamines to methanol, from acetate to methanol, and from acetate to trimethylamine, and the regulation of expression in the presence of multiple substrates. Our data provide new insight into the roles of multiple gene copies in methanoarchaea.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *Escherichia coli* cells were grown under standard conditions (31). *Methanosarcina* strains were grown with single-cell morphology (25) at 37°C in high-salt (HS) broth containing 125 mM methanol, 50 mM TMA, 50 mM DMA, 50 mM MMA, or 120 mM acetate, as appropriate (18). Growth on media solidified with 1.5% agar was as described by Boccazzi et al. (3). All plating manipulations were carried out under strictly anaerobic conditions in an anaerobic glove box. Plates containing solid media were incubated in an intrachamber anaerobic incubator as described previously (19). Puromycin (CalBiochem, San Diego, CA) was added from sterile, anaerobic stocks at a final concentration of 2 μg/ml for selection of *Methanosarcina* strains carrying puromycin transacetylase (*pac*). The purine analog 8-aza-2,6-diaminopurine (Sigma, St. Louis, MO) was added from sterile, anaerobic stocks at a final concentration of 20 μg/ml for selection against the *hpt* gene.

Methanosarcina strains used in the study were constructed by markerless gene exchange as described previously (22). All of the strains used are derivatives of *M. acetivorans* DSM 2834. WWM12 (*hpt*::P*mtaCB1*::*uidA*) was made using pMP58, WWM11 (*hpt*::P*mtaCB2*::*uidA*) was made using pMP59, WWM62 (hpt::P*mtaCB3*::*uidA*) was made using pMR52, and WWM63 (*hpt*::P*mcrB*::*uidA*) was made using pMR53. The plasmids used and their construction are summarized in Table 1.

Growth conditions for reporter gene assays. For the single-time reporter gene assays, the strains were grown on substrates for at least 25 generations (at least five transfers using 1% inocula) and then harvested at mid-exponential phase (optical density at 600 nm [OD_{600}] for TMA or methanol, 0.4 to 0.5; OD_{600} for DMA, 0.3; OD_{600} for acetate and MMA, 0.1 to 0.2) for β-glucuronidase assays. For the substrate shift experiments cultures were grown for at least 25 generations on TMA or acetate, harvested at the mid-exponential phase of growth by

TABLE 1. Plasmids used in this study

Plasmid	Description and/or construction	Reference
pWM356	pBluescript KS with the BamHI site filled	This study
pMP42	Vector for insertion into *hpt* locus	22
pJK41	Ap^r Pm^r cloning vector, *oriR6K* replicons	16
pMP45	NdeI/BglII-cut *uidA* PCR product [obtained using primers uidA(NdeI) and uidA(Bgl2)] cloned into BglII/BamHI-cut pWM368	22
pMP30	SpeI/MluI-digested up-*mtaC1* PCR product (obtained using primers SpeI2mtaC1 and MluINdeICI) and MluI/NotI-digested dn-*mtaC1* PCR product (obtained using primers MluIBamHB1 and NotI2mtaB1) cloned into SpeI/NotI-digested pJK41	21
pMP31	SpeI/MluI-digested up-*mtaC1* PCR product (obtained using primers SpeI2mtaC2 and MluINdeIC2) and MluI/NotI-digested dn-*mtaC1* PCR product (obtained using primers MluIBamHB2 and NotI2mtaB2) cloned into SpeI/NotI-digested pJK41	21
pMP38	SpeI/NotI-digested ΔCB1 fragment of pMP30 cloned into SpeI/NotI-digested pBluescript (-BamHI)	This study
pMP39	SpeI/NotI-digested ΔCB2 fragment of pMP30 cloned into SpeI/NotI-digested pBluescript (-BamHI)	This study
pMP51	NdeI/BglII *uidA* fragment from pMP45 cloned into NdeI/BamHI-digested pMP38	This study
pMP52	NdeI/BglII *uidA* fragment from pMP45 cloned into NdeI/BamHI-digested pMP39	This study
pMP58	SpeI/NotI fragment of pMP51 carrying P*mtaCB1-uidA* cloned into AvrII/NotI fragment of pMP42	23
pMP59	SpeI/NotI fragment of pMP51 carrying P*mtaCB2-uidA* cloned into AvrII/NotI fragment of pMP42	This study
pAMG46	Derivative of pMP42 with different cloning sites	23
pMR51	NotI/NdeI fragment of pMP58 cloned into NotI/NdeI-digested pAMG46	This study
pMR52	NheI/NdeI-digested P*mtaCB3* PCR product (obtained using primers oCB3/5′ and oCB3/3′) cloned into NheI/NdeI-digested pMR51	This study
pMR53	NheI/NdeI-digested P*mcrB* PCR product [obtained using primers oPmcrB(ac)/5′ and oPmcrB(ac)/3′] cloned into NheI/NdeI-digested pMR51	This study
pWM368	*Methanosarcina barkeri* Fusaro *mcrB* promoter source	32

centrifugation, washed two times with plain HS medium to remove residual substrate, and then resuspended in HS medium (500 ml) with the appropriate substrate(s) to an initial OD_{420} of 0.1. At various times, samples were withdrawn (30-ml samples during the lag phase of growth and 10-ml samples during the exponential phase of growth for acetate substrate shift experiments; 20-ml samples during the lag phase of growth and 10-ml samples during the exponential phase of growth for TMA substrate shift experiments) to measure growth (OD_{420}), the amount of methane produced, the methanol, TMA, DMA, and MMA concentrations, and the β-glucuronidase activity.

DNA methods. Standard methods were used throughout this study for isolation and manipulation of plasmid DNA from *E. coli* (1). The plasmids and primers used in this study are shown in Tables 1 and 2. Genomic DNA from *M. acetivorans* was isolated as described previously (22). DNA hybridization was performed using the DIG system (Roche, Mannheim, Germany). Magnagraph nylon transfer membranes were obtained from Osmonics (Westborough, MA). DNA sequences were determined using double-stranded templates by the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois.

Transformation methods. *E. coli* strains were transformed by electroporation using an *E. coli* Gene Pulser (Bio-Rad, Hercules, CA) as recommended by the supplier. Liposome-mediated transformation was used for *Methanosarcina* as described previously (3, 17).

Extract preparation and β-glucuronidase assay. The method used for preparation of cell extracts and the β-glucuronidase assay method were methods described previously (23).

Determination of metabolites. Methanogenic substrates and products were quantified using a Hewlett-Packard gas chromatograph (5890 Series II) equipped with a flame ionization detector. A stainless steel 80/120 Carbopack B column [Sigma-Aldrich (Supelco), St. Louis, MO] with He as the carrier gas was used at a constant temperature of 120°C for determination of methane and methanol contents. A glass 60/80 Carbopack B column [Sigma-Aldrich (Supelco), St. Louis, MO] with He as the carrier gas was used at a constant temperature of 95°C for measurement of TMA, DMA, and MMA. For analysis of TMA, DMA, and MMA, the samples were diluted 1:2 in 1% KOH before injection into the gas chromatograph. The column was washed with 10 μl distilled water, followed by 10 μl 1% KOH, between runs.

RESULTS

Construction of P*mtaCB1*::*uidA*, P*mtaCB2*::*uidA*, and P*mtaCB3*::*uidA* gene fusions. The *uidA* gene of *E. coli* encodes the easily assayable enzyme β-glucuronidase, which has previously been

TABLE 2. Primers used in this study

Primer	Sequence[a]	Added site(s)
SpeI2mtaC1	CCGCCGACTAGTTTATCTTTTTCAACCAGGGTAAGG	SpeI
MluINdeICI	CCGCCGACGCGTCATATGTCTAAACCTCCATTTAG	MluI, NdeI
MluIBamHB1	CCGCCGACGCGTGGATCCGCCCTCAGTTCTCTTTTTC	MluI, BamHI
NotI2mtaB1	CCGCCGGCGGCCGCCAGGAGGGATGGAAAAAGG	NotI
SpeI2mtaC2	CCGCCGACTAGTTTCAGCCTTCCAGAAAAACC	SpeI
MluINdeIC2	CCGCCGACGCGTCATATGTTAACCTCCATTTTAATAATGAAGC	MluI, NdeI
MluIBamHB2	CCGCCGACGCGTGGATCCAATTTTTTTTCAAAAAATGGC	MluI, BamHI
NotI2mtaB2	CCGCCGGCGGCCGCGTCCTGCATACGAGATGTTCC	Not
uidA(NdeI)	GGGGGGCATATGTTACGTCCTGTAGAAACCC	NdeI
uidA(Bgl2)	GGGGGGAGATCTGATCATTAACGGCGCAGTACCG	BglII
oCB3/5′	GGAATTCCATATGCTAGCTAGCCGCGATAGATATTTGAAAAACATCTATC	NdeI, AceII
oCB3/3′	GAATTCCCATATGTTTAACCTCCATTTTAGTATTTGAGGAGTAAATATC	NdeI
oPmcrB(ac)/5′	GGAATTCCATATGCTAGCTAGCCAGAGGGTCTTTTCGAGGAC	NdeI, AceII
oPmcrB(ac)/3′	GAATTCCCATATGAATTTCCTCCTTAATTTATTAAAATCATTTTGGG	NdeI

[a] Added restriction sites are underlined.

TABLE 3. β-Glucuronidase activities of *uidA* translational fusions to *mtaCB1*, *mtaCB2*, *mtaCB3*, and *mcrB* in cells grown on various methanogenic substrates[a]

Fusion	β-Glucuronidase activities with the following substrates:				
	Methanol	TMA	DMA	MMA	Acetate
mtaC1[b]	315 ± 47	3.3 ± 0.3	1.14 ± 0.2	1.1 ± 0.1	3.6 ± 0.7
mtaC2	1,666 ± 140	2.9 ± 0.8	1.9 ± 0.2	15 ± 2.7	128 ± 28
mtaC3	25 ± 6	36 ± 5	40 ± 3	161 ± 40	241 ± 19
mcrB	548 ± 53	802 ± 65	432 ± 55	605 ± 31	2,159 ± 179

[a] Activity is expressed in mU/mg protein (nmol min^{-1} mg $protein^{-1}$). The values are averages and standard errors for nine independent measurements.
[b] Data for this fusion were originally reported by Rother et al. (23) and are included for completeness.

used as a reporter gene in *Methanosarcina* and other methanoarchaea (2, 12, 23). We constructed *uidA* reporter gene fusions to the promoter regions of the *mtaCB1* (23), *mtaCB2*, and *mtaCB3* operons. In these constructs the start codon of the *uidA* gene was superimposed with the start codon of the corresponding *mtaC* gene, and the constructs carried ca. 1 kb of upstream DNA. However, the *mtaC3* gene apparently utilizes a TTG start codon (21), which was changed to ATG in the P*mtaC3*::*uidA* fusion to facilitate the cloning steps and to maintain uniformity with the other fusions. As a control, we utilized a fusion to the promoter of the *mcrB* gene of *M. acetivorans* C2A, which is the first gene of the *mcrBDCGA* operon. This operon encodes methyl-coenzyme M reductase, which catalyzes the terminal step of methanogenesis during growth on all methanogenic substrates. It should be noted that the promoters for these operons have not been precisely mapped. We assumed that all required regulatory sequences were present within the 1-kb region upstream of the coding region because all three *mtaCB* fusions were expressed on one or more substrates, although it is possible that additional control sequences were present, either further upstream or within the coding sequence. Each of the fusion constructs was subsequently integrated as a single copy into the *hpt* locus on the *M. acetivorans* C2A chromosome using the markerless exchange method (22). All strains were verified to have the correct insertions into the *hpt* locus by DNA hybridization (data not shown).

Expression of *mtaCB* operons on various methanogenic substrates. As described above, our previous gene deletion experiments suggested discrete roles for each of the three *mtaCB* operons, which might be reflected in gene regulation. Thus, we examined the expression of the three *mtaCB*::*uidA* fusions at mid-exponential phase during growth in media containing either methanol, TMA, DMA, MMA, or acetate (Table 3). The *mtaCB1* and *mtaCB2* promoter gene fusions were highly upregulated on methanol compared to the expression with the other substrates tested. The *mtaCB1* promoter gene fusion was induced ~100-fold during growth on methanol (compared to expression on TMA), whereas the *mtaCB2* promoter gene fusion was induced ~575-fold. These data strongly support previous molecular, genetic, and biochemical data suggesting that both operons are specifically involved in the metabolism of methanol. Interestingly, the *mtaCB1* promoter gene fusion was expressed only during growth on methanol, while expression of the *mtaCB2* promoter gene fusion was also induced during growth on both acetate and MMA. Thus, despite the observation that both promoter gene fusions are expressed during growth on methanol, the two operons are clearly differentially regulated. In contrast, expression of the *mtaCB3* promoter gene fusion was not induced in response to methanol at mid-exponential phase. Instead, this promoter gene fusion appeared to be expressed at a low, constitutive level during growth on methanol, TMA, or DMA. However, expression of the *mtaCB3* promoter gene fusion increased ~10-fold during growth on acetate and ~5-fold during growth on MMA (relative to expression on TMA) (Table 3). Although the expression of the control *mcrB* promoter gene fusion also increased during growth on acetate, the level of induction (approximately threefold) was much less than the expression of the *mtaCB3* promoter gene fusion. Furthermore, the control promoter gene fusion was not induced on MMA. Thus, the expression pattern of the *mtaCB3* promoter gene fusion appears to suggest that there is substrate-specific gene regulation that is clearly different than that of the *mtaCB1* and *mtaCB2* promoter gene fusions. The increased expression of the *mtaCB3* promoter gene fusion suggests that these genes might play an important role during growth on acetate, despite the observation that *mtaCB3* mutants do not have any known phenotype on acetate (21).

Growth phase-dependent expression of *mtaCB* isozymes. In an effort to further delineate the regulatory differences between *mtaCB1*, *mtaCB2*, and *mtaCB3*, the temporal expression pattern of each promoter gene fusion strain was analyzed when TMA-adapted cultures were switched to methanol (Fig. 2). In addition, substrate shift experiments were performed by shifting acetate-grown cultures to either methanol (Fig. 3) or TMA (data not shown). At each time, reporter gene activity, growth (OD_{420}), methanol consumption, and methane production (data not shown) were also measured.

A significant lag occurred prior to expression of any of the reporter gene fusions. None of the *mtaCB* promoter gene fusions were expressed during most of this period (75 h for *mtaCB1* and *mtaCB3* and 50 h for the *mtaCB2* promoter), which argues against a simple model of transcriptional regulation involving a methanol-sensing repressor protein. The *mtaCB1* promoter gene fusion was expressed early in exponential growth, but only after methanol utilization and methane production (data not shown) had already begun. This indicates that another methanol-specific MT1 (either MtaCB2 or MtaCB3 or both) was responsible for the activation of methanol during the earliest phase of growth. Both the *mtaCB2* and *mtaCB3* promoter gene fusions were expressed during this preexponential phase of growth, before significant methanol utilization, methane production (data not shown), or cell

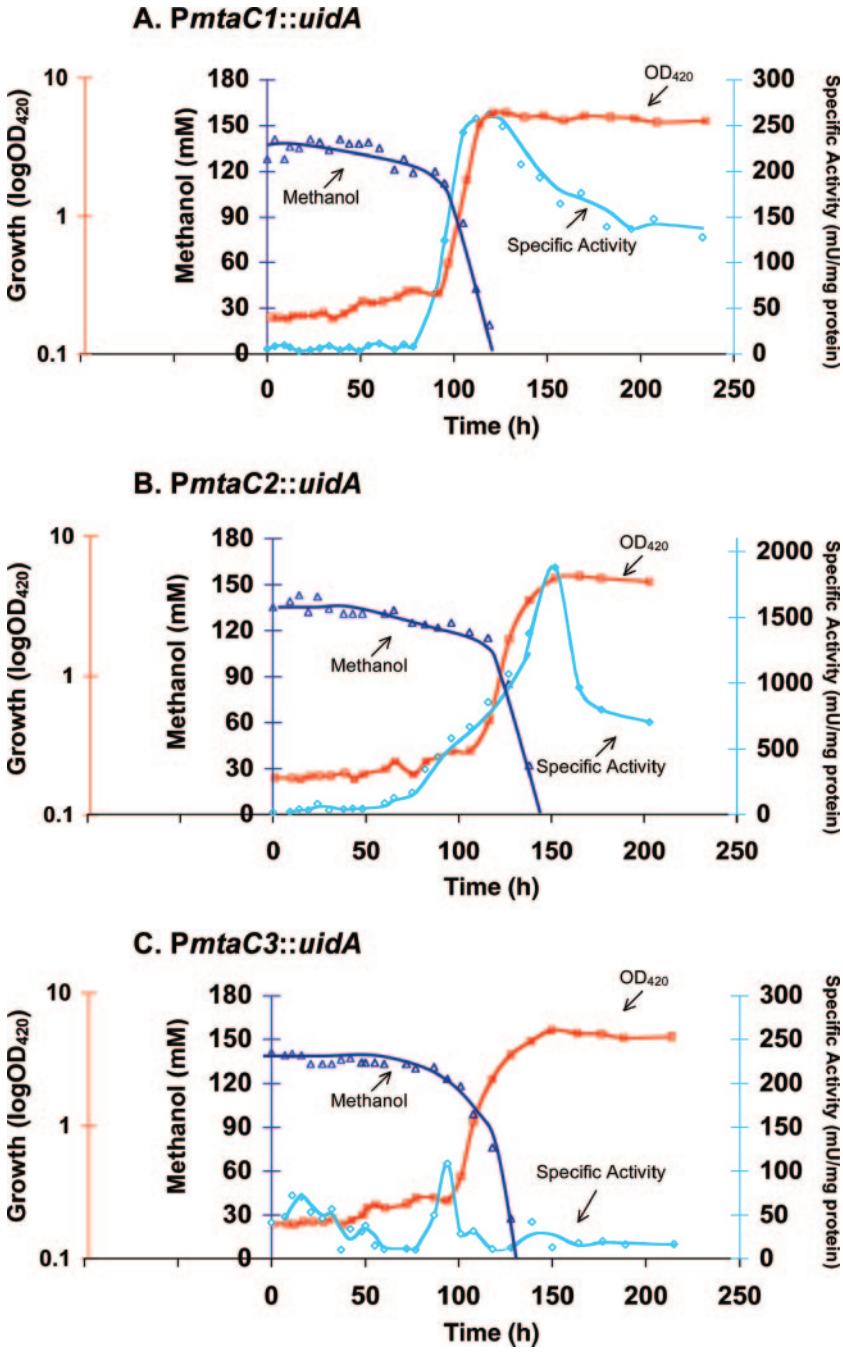

FIG. 2. P*mtaC*::*uidA* expression during a substrate shift from trimethylamine to methanol. Cultures adapted to TMA (50 mM) were switched to medium containing methanol (125 mM). The OD_{420} indicates growth, and the methanol concentration was determined by gas chromatography. The β-glucuronidase activity indicates expression from the specific promoters. (A) P*mtaC1*::*uidA*; (B) P*mtaC2*::*uidA*; (C) P*mtaC3*::*uidA*. The *y* axes indicate log OD_{420} (red), methanol concentration (dark blue), and β-glucuronidase activity (light blue). The β-glucuronidase activities of the three strains were very different, and thus the scales used for specific activity are not identical. Triplicate experiments were performed for each fusion strain. Equivalent results were obtained for all replicates; a representative curve is shown for each experiment.

growth was observed. Although induction of the *mtaC3* promoter gene fusion was transient during the TMA-to-methanol substrate shift experiment, the level of expression was significant (at least threefold above the basal level of expression) and reproducible in all replicates of this experiment, suggesting a specific role for *mtaCB3* during this substrate shift. The induction of the *mtaCB3* promoter gene fusion was not clear during the acetate-to-methanol substrate shift experiment because this operon was induced by pregrowth on acetate. Expression of both the *mtaCB1* and *mtaCB2* promoter gene fusions peaked during early stationary phase.

During the acetate-to-TMA substrate shift experiment (data

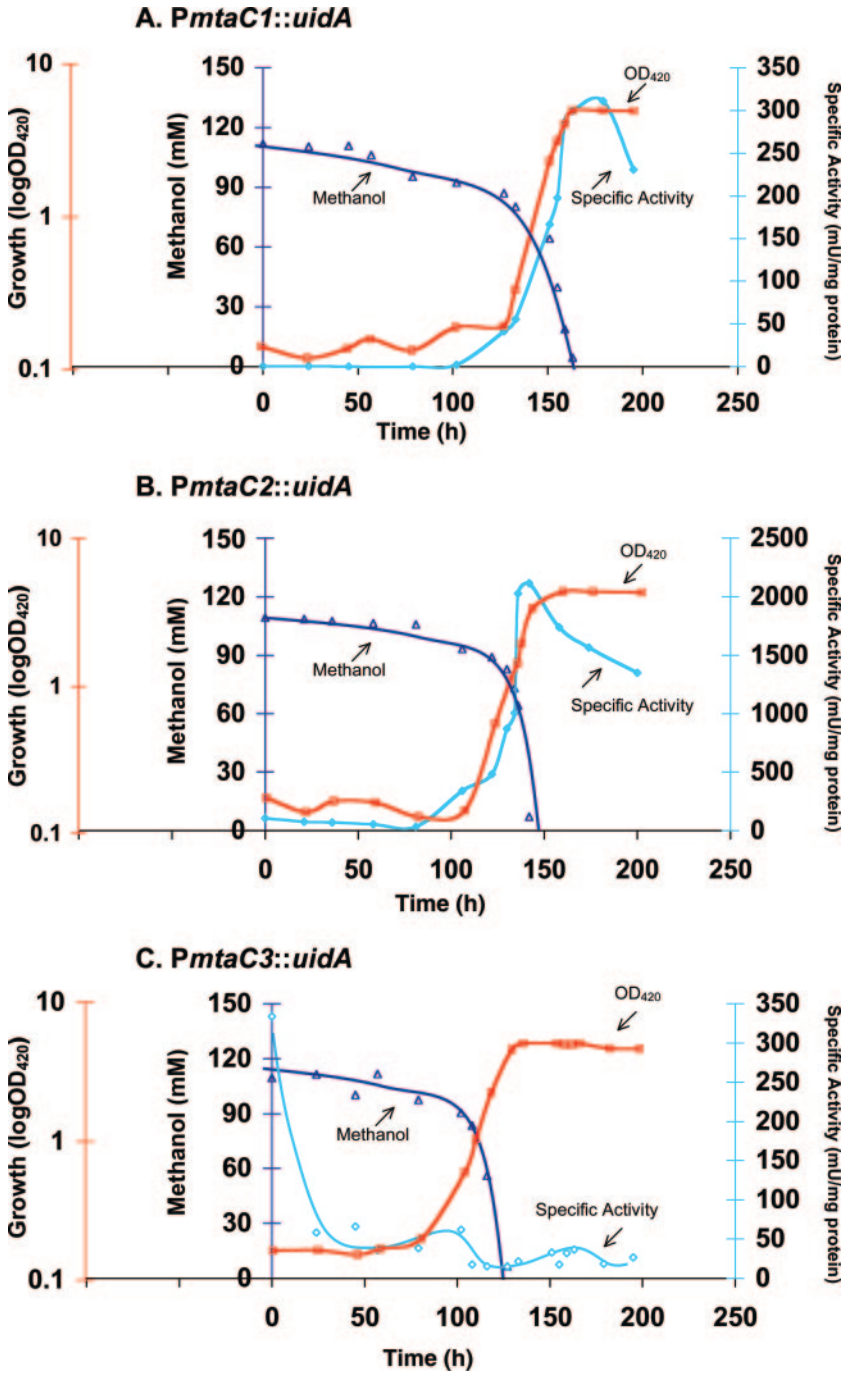

FIG. 3. P*mtaC*::*uidA* expression during a substrate shift from acetate to methanol. Cultures adapted to acetate (120 mM) were switched to medium containing methanol (125 mM). The OD_{420} indicates growth, and the methanol concentration was determined by gas chromatography. The β-glucuronidase activity indicates expression from the specific promoters. (A) P*mtaC1*::*uidA*; (B) P*mtaC2*::*uidA*; (C) P*mtaC3*::*uidA*. The *y* axes indicate log OD_{420} (red), methanol concentration (dark blue), and β-glucuronidase activity (light blue). The β-glucuronidase activities of the three strains were very different, and thus the scales used for specific activity are not identical. Duplicate experiments were performed for each fusion strain. Equivalent results were obtained for all replicates; a representative curve is shown for each experiment.

not shown) the *mtaCB1* promoter gene fusion was not expressed at any time during growth. However, both the *mtaCB2* and *mtaCB3* promoter gene fusions were expressed later during growth. The expression of the *mtaCB2* promoter gene fusion increased when DMA appeared in the medium, whereas the expression of the *mtaCB3* promoter gene fusion increased when MMA appeared in the medium, which is consistent with the observations from the mid-exponential measurements of expression of these promoters (Table 3).

Expression of *mtaCB* operons in the presence of both TMA and methanol. To examine the effect of the presence of multiple substrates on the expression of the three *mtaCB* promoter

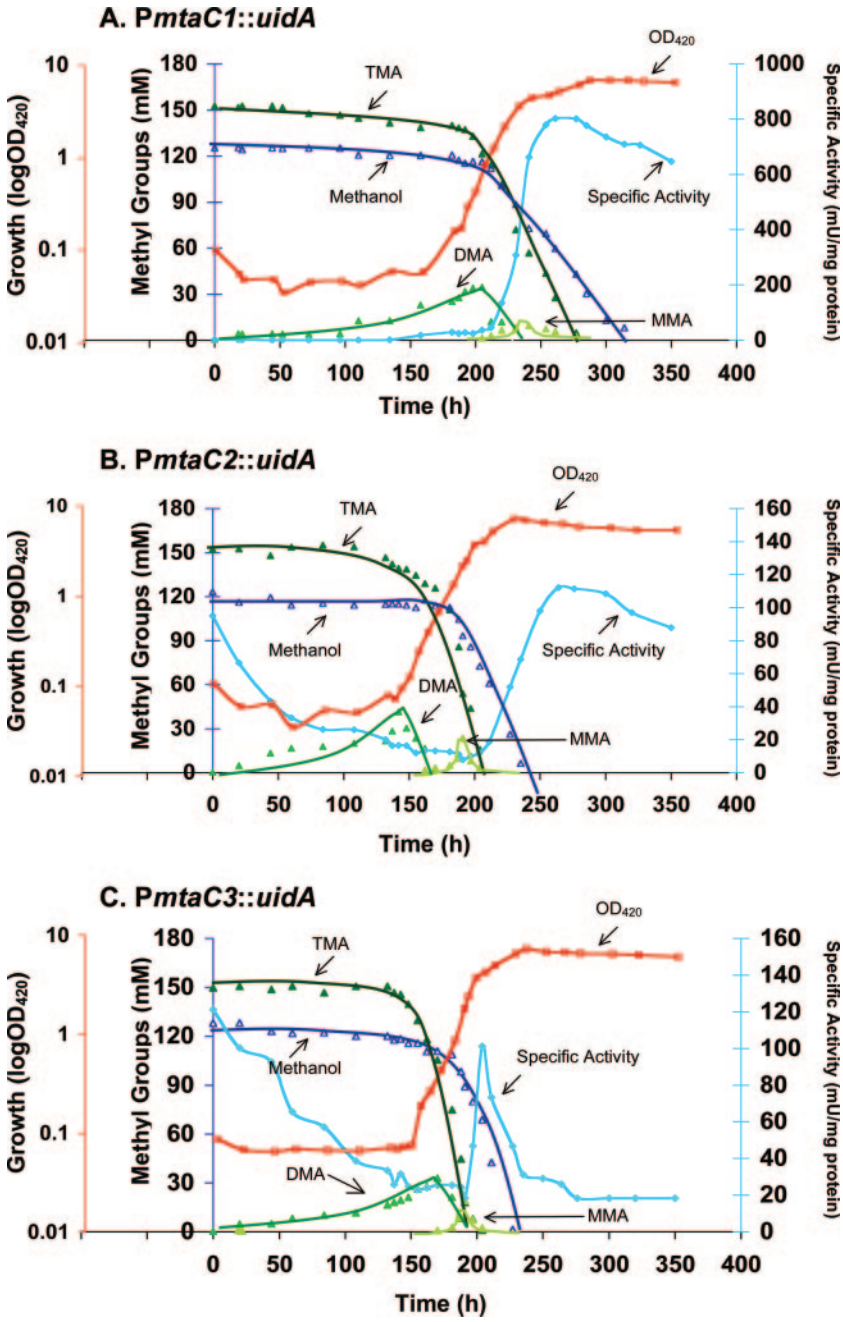

FIG. 4. P*mtaC*::*uidA* expression during a substrate shift from acetate to methanol and trimethylamine. Cultures adapted to acetate (120 mM) were switched to medium containing both methanol (125 mM) and TMA (50 mM). The OD_{420} indicates growth, and the methanol, TMA, DMA, and MMA concentrations were determined by gas chromatography. The β-glucuronidase specific activity indicates expression from the specific promoters. (A) P*mtaC1*::*uidA*; (B) P*mtaC2*::*uidA*; (C) P*mtaC3*::*uidA*. The *y* axes indicate log OD_{420} (red), methanol concentration (dark blue), and β-glucuronidase activity. The β-glucuronidase activities of the strains were very different, and thus the scales used for specific activity are not the same. Duplicate experiments were performed for each fusion strain. Equivalent results were obtained for both replicates; a representative curve is shown for each experiment.

gene fusions, cultures were grown to mid-exponential phase in acetate medium and then shifted to a medium containing both TMA and methanol (Fig. 4). Reporter gene activity, growth, methane production (data not shown), and the concentrations of methanol, TMA, DMA, and MMA were monitored throughout the experiment. (DMA and MMA are the products of TMA utilization and are themselves substrates for growth and methanogenesis.)

Two significant trends were observed in the substrate shift experiment in which there was a shift from acetate to a combination of TMA and methanol (Fig. 4). First, we observed that the cells used TMA almost exclusively for initial growth

before any detectable decrease in the methanol concentration. The early accumulation of DMA in the medium supports the hypothesis that there was early utilization of TMA for growth. Both DMA and MMA started accumulating before rapid utilization of methanol commenced. Finally, the growth curve displays a diauxic pattern (which is more pronounced when the data are plotted on a linear scale), which correlates with the nearly simultaneous depletion of TMA, DMA, and MMA. In keeping with the idea that TMA is preferred over methanol for initial growth, there was no observable induction of any of the *mtaCB* promoter gene fusions during the initial stages of growth. (Note that the expression of *mtaCB2* and *mtaCB3* promoter gene fusions, which was induced by pregrowth on acetate, actually declined during the lag phase prior to initiation of growth.) Second, the relative order and degree of expression of the *mtaCB* promoter gene fusions were substantially altered during the substrate shift experiment in which there was a shift from acetate to a combination of methanol and methylamines. As described above, both the *mtaCB2* and *mtaCB3* promoter gene fusions were expressed prior to the expression of the *mtaCB1* promoter gene fusion when cells were shifted from either TMA or acetate to methanol (Fig. 2 and 3). In contrast, when cells were switched from acetate to the mixture of TMA and methanol, the *mtaCB1* promoter gene fusion was expressed well before the *mtaCB2* promoter gene fusion was expressed. Furthermore, the level of expression of the *mtaCB1* promoter gene fusion was approximately threefold higher during the switch from acetate to TMA plus methanol than during the switch from TMA to methanol. It is also notable that neither the *mtaCB2* promoter gene fusion nor the *mtaCB3* promoter gene fusion was induced until ca. 90% of the TMA had been utilized. Moreover, the level of expression of the *mtaCB2* promoter gene fusion with the combination of TMA and methanol was only 5% of the expression level observed after the switch from TMA to methanol. (It should be noted that in the TMA-to-methanol substrate shift experiment the cells were washed twice prior to transfer to remove residual methylamines, ensuring that their presence did not affect expression of the three *mtaCB* promoter gene fusions.) In addition, the induction of the *mtaCB3* promoter gene fusion was concurrent with the accumulation of MMA in the medium, which was in accordance with the earlier observation that the expression of the *mtaCB3* promoter gene fusion was induced on MMA.

DISCUSSION

The data presented here clearly show that the *mtaCB1*, *mtaCB2*, and *mtaCB3* promoter gene fusions are differentially regulated in *M. acetivorans*, strongly supporting the idea that the three MT1 methyltransferases play discrete roles in the cell. In the present study we showed that *mtaCB1* is expressed only during exponential growth on methanol. This is consistent with the purification of MtaCB1 as the sole MT1 purified from methanol-grown *M. barkeri* (24). In addition, our previous observation shows that MtaCB1 has the highest methyltransferase activity (21). Based on these data, we suggest that MtaCB1 is the primary MT1 utilized during exponential growth on methanol. The roles of MtaCB2 and MtaCB3, however, are somewhat more difficult to define. It has been proposed that prior synthesis of MtaCB3 during growth on acetate allows efficient switching from acetate to methanol (6, 15). Our finding that *mtaCB3* is expressed on all substrates is consistent with this idea. Moreover, the transient induction of this operon right at the point of switching to methanol, but not at the point of switching to TMA from acetate (Fig. 2), supports a specific role in substrate switching to methanol. However, it should be remembered that mutants lacking *mtaCB3* have shorter lag times when they are switched from TMA to methanol than mutants lacking either *mtaCB1* or *mtaCB2* have (21). Thus, *mtaCB3* is not absolutely required for switching, nor does MtaCB3 appear to be the only enzyme involved. We suggest that *mtaCB2* probably also plays a significant role in substrate switching. This idea is consistent with the long lag phase during switching to methanol that is observed in *mtaCB2* mutants (21) and with the observation made here that, in the absence of substrates other than methanol, *mtaCB2* is the first of the three operons to be expressed. However, the highest level of *mtaCB2* and *mtaCB1* expression is observed as the cells enter stationary phase. This suggests the possibility that the cells recognize methanol depletion and accumulate high levels of MtaCB2 and MtaCB1 in order to be prepared for rapid growth onset should the substrate become available again. An alternative, and equally consistent, model involves MtaCB2 as a high-affinity (low-K_m) methanol-activating enzyme. Thus, as the concentration of methanol falls during growth in batch culture, the need for a high-affinity enzyme and correspondingly the expression of *mtaCB2* increase, reaching the maximum level as the cells enter stationary phase. In addition, if induction of a methanol transporter were required, the operon would be expressed early in growth. Thus, prior to expression of this putative transporter, cytoplasmic methanol concentrations would be low, requiring the low-K_m enzyme, explaining the prolonged lag phase of *mtaCB2* mutants. Because of the trade-off between high affinity and enzyme velocity (low V_{max}), this would also explain the low methyltransferase activity of strains expressing only MtaCB2 (21), which is especially puzzling given the very high levels of expression of the *mtaCB2* reporter gene fusion (almost 10-fold higher than the levels of expression of *mtaCB1* [Table 3]).

Our data also clearly show that regulation of *mtaCB* operons is more complex than a simple response to methanol and growth phase. In the substrate shift experiment in which there was a shift from acetate to methanol plus TMA, expression of *mtaCB2* and *mtaCB3* did not occur until ca. 90% of the methylamine was consumed. Therefore, it appears that methylamines might inhibit expression of *mtaCB2* and *mtaCB3* operons, although to different degrees. It is also interesting that both the *mtaCB2* and *mtaCB3* operons are specifically induced during growth on MMA (Table 3 and Fig. 4) and are induced even more on acetate (Table 3). Both methanol and TMA have been shown to be important substrates for methanogens in marine sediments (20). Thus, in order to understand the preferential utilization of TMA over methanol for initial growth, we turned to the energetics of methanogenesis from various methanogenic substrates. It has been argued that the low energy yield available from methanogenesis from acetate requires the prior synthesis of the enzymes needed for use of a new substrate in order to promote efficient switching (6). We believe that the same argument would hold for methanogen-

TABLE 4. Free energy of methanogenic reactions[a]

Substrate	Reaction	$\Delta G^{\circ\prime}$ (kJ)		
		Per reaction	Per mol CH_4	Per mol substrate
Methanol	$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	−318	−106	−80
TMA	$4(CH_3)_3N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3$	−682	−76	−171
DMA	$2(CH_3)_2NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3$	−224	−75	−112
MMA	$4CH_3NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3$	−230	−77	−57
Acetate	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	−36	−36	−36

[a] Values were calculated from data of Thauer et al. (29).

esis from MMA. However, this must be examined carefully. Typically, the energetics of methanogenesis are reported as $\Delta G^{\circ\prime}$/mol CH_4 (Table 4). On this basis, acetate is clearly the poorest substrate, TMA, DMA, and MMA are about equal, and methanol is the best substrate. This is a useful measure, in that it reveals the free energy available from electron transport on a per-electron basis. Moreover, the values are consistent with the observation that the highest growth rates are obtained during growth on methanol (21). Nevertheless, when substrate preference is considered, it is probably much more relevant to compare the $\Delta G^{\circ\prime}$/mol substrate. On this basis TMA is the best substrate by a wide margin, followed by DMA, methanol, MMA, and finally acetate. With this in mind, our data are consistent with hierarchical regulation of the MT1-encoding genes based on the quality of the available substrates, analogous to catabolite repression in enteric bacteria. This model readily explains the preference for TMA over methanol in the substrate shift experiment, the lack of expression of the *mtaCB* operons in the presence of TMA, and the induction of the putative switching enzymes (MtaCB3 and MtaCB2) during growth on low-energy substrates.

At present, it is unclear how the complex differential regulation of the *mtaCB* operons is achieved, but it seems clear that multiple types of information must be integrated. Our data eliminate many of the simple regulatory paradigms. For example, a methanol-sensing activator or repressor protein alone does not explain the low level of expression of the operons when both methanol and TMA are present. Nor does a TMA-sensing repressor explain the specific induction of *mtaCB3* and *mtaCB2* on MMA and acetate. Further examination of this process is clearly warranted, and such studies are being performed in our laboratory. In this regard, the *mtaCB* operons may be among the most fertile candidates for study of gene regulation in archaea. To our knowledge, the level of regulation measured in our *mtaCB1* and *mtaCB2* fusions is unprecedented in the archaeal domain. We have recently shown that it is possible to select constitutive mutants by fusing the *mtaC1* promoter to an essential gene (23), suggesting that genetic approaches should be fruitful in unraveling this mystery.

Finally, given the wealth of data coming from proteomic and microarray projects, one might question the need for single-promoter reporter gene studies. Indeed, our data are qualitatively consistent with the *M. acetivorans* proteomic data obtained by Li et al. (14, 15), although the fold regulation that we observed is much higher. Because the proteomic studies rely on quantification of stained protein spots on a two-dimensional gel, the values must be considered minimum estimates. Since our fusions maintain both transcriptional and translational control sequences, we believe that the reporter genes provide a more accurate reflection of the actual degree of *mtaCB* gene regulation. Our data are, however, at odds with the results obtained with *M. mazei* microarrays (11). Although it is possible that the data reflect real differences in gene regulation between the two species, we feel that it is likely that the difficulties in analysis of highly homologous genes may account for the discrepancies. Accordingly, it seems very likely that the multiple *mtaCB* operons cross-hybridize during nucleic acid hybridization experiments. This problem is well recognized and was acknowledged by the authors of the *M. mazei* study (4, 11, 13). The genetic approach used here has no difficulty in discriminating between the different gene copies. Moreover, the dynamic studies described here, although technically possible, would be extremely costly and time-consuming using either microarray or proteomic approaches. In contrast, the reporter gene approach used here is rapid, simple, and inexpensive. However, reporter gene fusions have a few disadvantages compared to microarray analysis. In particular, although gene fusions can readily be used to show activation of expression, they have limited capacity for demonstrating gene shutoff (due to the stability of the reporter protein). In addition, it is formally possible that introduction of an additional copy of the promoter under study may perturb the regulatory system. With these caveats in mind, our studies revealed differential and timely regulation of important genes in the methanol utilization pathway, showing the complex interaction between multiple substrates and growth phases and providing new insight into the roles of multiple copies of many methanogenesis genes in *Methanosarcina*.

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