AEM
Journals.ASM.org

Genes of the *N*-Methylglutamate Pathway Are Essential for Growth of *Methylobacterium extorquens* DM4 with Monomethylamine

Christelle Gruffaz,[a] Emilie E. L. Muller,[a] Yousra Louhichi-Jelail,[a] Yella R. Nelli,[b] Gilles Guichard,[b] Françoise Bringel[a]

Université de Strasbourg, UMR 7156 CNRS, Génétique Moléculaire, Génomique, Microbiologie, Strasbourg, France[a]; Université de Bordeaux, UMR 5248 CNRS, Institut Polytechnique de Bordeaux, CBMN, Institut Européen de Chimie Biologie, Pessac, France[b]

Monomethylamine (MMA, CH_3NH_2) can be used as a carbon and nitrogen source by many methylotrophic bacteria. *Methylobacterium extorquens* DM4 lacks the MMA dehydrogenase encoded by *mau* genes, which in *M. extorquens* AM1 is essential for growth on MMA. Identification and characterization of minitransposon mutants with an MMA-dependent phenotype showed that strain DM4 grows with MMA as the sole source of carbon, energy, and nitrogen by the *N*-methylglutamate (NMG) pathway. Independent mutations were found in a chromosomal region containing the genes *gmaS*, *mgsABC*, and *mgdABCD* for the three enzymes of the pathway, γ-glutamylmethylamide (GMA) synthetase, NMG synthase, and NMG dehydrogenase, respectively. Reverse transcription-PCR confirmed the operonic structure of the two divergent gene clusters *mgsABC-gmaS* and *mgdABCD* and their induction during growth with MMA. The genes *mgdABCD* and *mgsABC* were found to be essential for utilization of MMA as a carbon and nitrogen source. The gene *gmaS* was essential for MMA utilization as a carbon source, but residual growth of mutant DM4*gmaS* growing with succinate and MMA as a nitrogen source was observed. Plasmid copies of *gmaS* and the *gmaS* homolog METDI4690, which encodes a protein 39% identical to GMA synthetase, fully restored the ability of mutants DM4*gmaS* and DM4*gmaS*Δmetdi4690 to use MMA as a carbon and nitrogen source. Similarly, chemically synthesized GMA, the product of GMA synthetase, could be used as a nitrogen source for growth in the wild-type strain, as well as in DM4*gmaS* and DM4*gmaS*Δmetdi4690 mutants. The NADH:ubiquinone oxidoreductase respiratory complex component NuoG was also found to be essential for growth with MMA as a carbon source.

Monomethylamine (MMA; methylamine) is a nitrogen-containing C_1 compound released by natural sources such as the breakdown of proteins and amine osmolytes, as well as by human-made nitrogen-containing pesticides, pharmaceuticals, and herbicides (1). MMA is ubiquitous in the environment and can serve as the sole source of carbon and energy for methylotrophic bacteria, which grow on compounds with no C-C bonds (2) but also as a nitrogen source for a large variety of bacteria (3). MMA utilization by Gram-negative bacteria occurs either by oxidation of MMA into formaldehyde by MMA dehydrogenase (MADH) encoded by *mau* genes (4) found only in methylotrophic bacteria so far (5) or by the *N*-methylglutamate (NMG) pathway, the genes for which were first identified in the betaproteobacterium *Methyloversatilis universalis* FAM5 (6). This metabolism effects the condensation of MMA with glutamate to NMG, with γ-glutamylmethylamide (GMA) a possible intermediate (Fig. 1). The genes *gmaS*, *mgsABC*, and *mgdABCD* encode GMA synthetase (GMAS), NMG synthase, and NMG dehydrogenase, respectively (6). Of these, only *gmaS* was found not to be required for MMA metabolism in *M. universalis* FAM5 (6). In contrast, *gmaS* was also required for MMA utilization in the facultative methane utilizer *Methylocella silvestris* BL2 (1). In addition, the NMG pathway was recently demonstrated to be also involved in nitrogen assimilation by nonmethylotrophic bacteria (7).

Both strains DM4 and AM1 of the alphaproteobacterial species *Methylobacterium extorquens* are able to use MMA as the sole source of carbon and nitrogen. However, comparative genomic analysis demonstrated that the *mau* cluster essential for strain AM1 to grow on MMA as the sole source of carbon and energy (4) was absent from strain DM4 (8). A bank of several thousand mutants obtained by random mutagenesis was used to identify genes required for MMA utilization as a carbon and nitrogen source by *M. extorquens* DM4, and MMA-dependent gene expression studies and targeted-site mutagenesis were performed. The role of the NMG pathway for MMA oxidation by *M. extorquens* DM4 was assessed by comparisons with other methylotrophic strains with previously characterized genes for MMA oxidation by the NMG pathway (1, 6) and with the closely related strain *M. extorquens* AM1, which also contains canonical *mau* genes for MMA dehydrogenase in addition to genes for the NMG pathway (4).

MATERIALS AND METHODS

Strains and growth conditions. *M. extorquens* DM4 was cultivated aerobically at 30°C in mineral medium M3 (9) unless specified otherwise. *Escherichia coli* strains DH5α and S17-1 (ATCC 47055) were cultivated aerobically at 37°C in Luria-Bertani medium (Difco Laboratories). M3N0 medium (N-depleted M3 medium) was used to assess the filter-sterilized nitrogen sources ammonium sulfate (1.5 mM), MMA (1.5 mM), and GMA (0.75 mM, i.e., 1.5 mM total nitrogen). Chemical synthesis of GMA was achieved by a protocol specifically developed for this study (see Fig. S1 in the supplemental material). Dichloromethane (Fluka) was added (300 μl) in a glass tube placed in a 3.3-liter glass jar, which was hermetically closed. Antibiotics were used at final concentrations of 10 $\mu g \cdot ml^{-1}$ (tetracycline) and 25 $\mu g \cdot ml^{-1}$ (kanamycin) as required.

Received 14 January 2014 Accepted 25 March 2014
Published ahead of print 28 March 2014
Editor: G. Voordouw
Address correspondence to Françoise Bringel, francoise.bringel@unistra.fr.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.04160-13.

doi:10.1128/AEM.04160-13

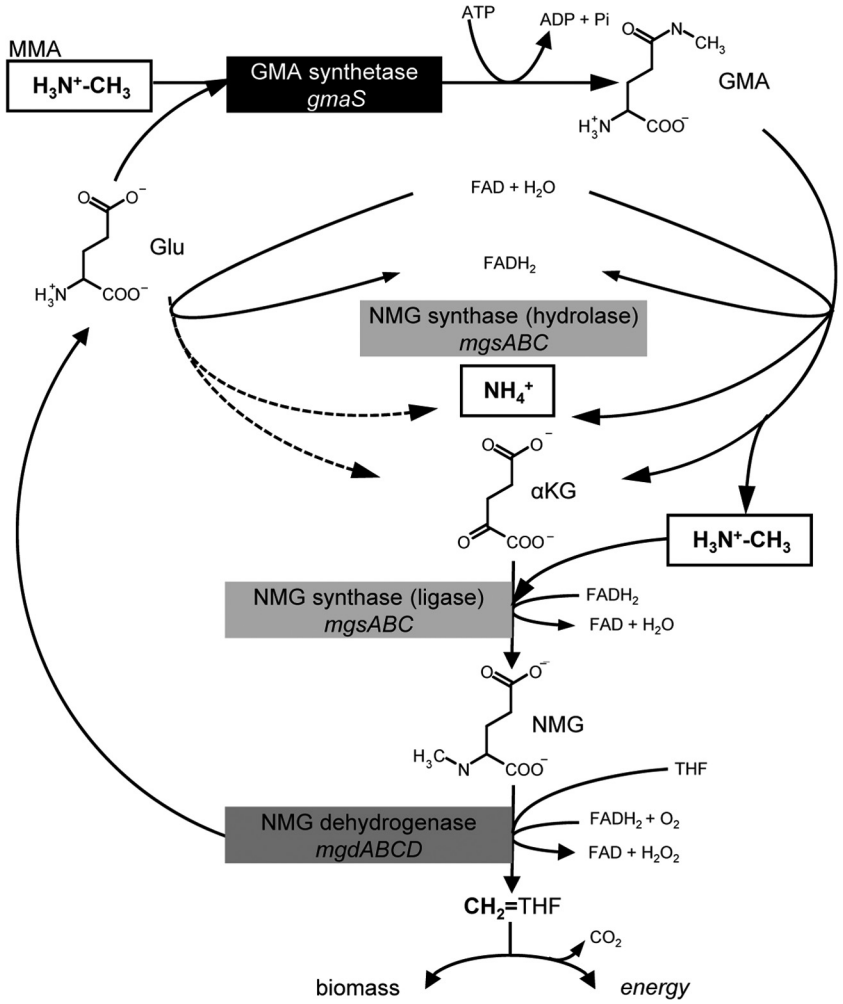

FIG 1 Proposed pathway and genes for MMA utilization as the source of carbon, energy, and nitrogen on the basis of observed mutant phenotypes of *M. extorquens* DM4. MMA oxidation by the NMG pathway involves GMAS (EC 1.6.5.3, encoded by *gmaS*), NMG synthase (EC 2.4.2.2, encoded by *mgsABC*), and NMG dehydrogenase (EC 1.5.99.5, encoded by *mgdABCD*). α-Ketoglutarate and tetrahydrofolate are abbreviated as αKG and THF, respectively. In *M. extorquens* DM4, GMAS is required for growth with MMA as the carbon source. In the absence of *gmaS*, direct oxidative deamination of glutamate by NMG synthase (hydrolase) may yield sufficient ammonium for growth (broken lines). NMG dehydrogenase catalyzes the transformation of NMG to methylene tetrahydrofolate, which subsequently enters the serine cycle for carbon assimilation into biomass or is oxidized into CO_2 for energy production via enzymes encoded by *mdtA*, *fch*, *ftfL*, and *fdh* (27). MMA utilization as a sole carbon and nitrogen source was shown to require the presence of both *mgs* and *mgd* (see the text).

Growth rates were determined as the average of at least two independent replicates in 20-ml liquid cultures in agitated (100 rpm) 100-ml Erlenmeyer flasks by measuring optical density at 600 nm (OD_{600}) spectrophotometrically, with the exception of cultures with GMA as the growth substrate, which were performed in 5 ml medium in 16-ml Hungate tubes, and the OD was monitored directly in the tubes (Libra S6; Biochrom).

Screening and mapping of minitransposon insertion mutants. The previously described mutant library (10) of random transcriptional *gfp* fusion minitransposon insertions in *M. extorquens* DM4 was conserved at −80°C in 96-well microtiter plates in M3 medium containing methanol, 25% glycerol, and kanamycin. Individual mutants were spotted onto solid M3 plates containing MMA at 20 mM, methanol at 20 mM, or both at 20 mM each, and their abilities to grow and emit fluorescence were tested after 5 days at 30°C. Control strains included the nonfluorescent wild-type strain, a mutant with constitutive expression of green fluorescent protein (GFP) carrying the minitransposon in coding sequence (CDS) METDI4743 of unknown function (10), and mutant DM4*cycH*, which is unable to grow with methanol and was selected and characterized during this work. Mutants with an MMA-dependent phenotype were then further assayed for growth and induction of GFP fluorescence in M3 medium containing MMA (20 and 80 mM), dichloromethane (1.4 mM), methanol or formate (20 mM), pyruvate or glycerol (7.5 mM), acetate or betaine (10 mM), succinate (5 mM), and combinations thereof. Growth and fluorescence were tested after 7 days of incubation at 30°C of 5-μl drops of serial dilutions of bacterial cultures by a two-step PCR method combining semidegenerate primers together with minitransposon-specific primers as previously described (10). The sequences obtained were compared with the DM4 genome sequence by using BLAST (11) to determine the minitransposon insertion site.

Site-direct mutagenesis. Wild-type and mutant alleles were reciprocally exchanged by using the *sacB*-based pCM433 vector for marker-free allelic exchange as previously described (12). Briefly, mutant alleles were constructed by a two-step PCR amplification process. The first step involved the use of two primer pairs to amplify approximately 0.5 kb upstream and downstream to the region targeted for whole CDS deletion (see right and left deletion primer pairs in Table S1 in the supplemental material). The two fragments, overlapping by 16-nucleotide (nt)-long

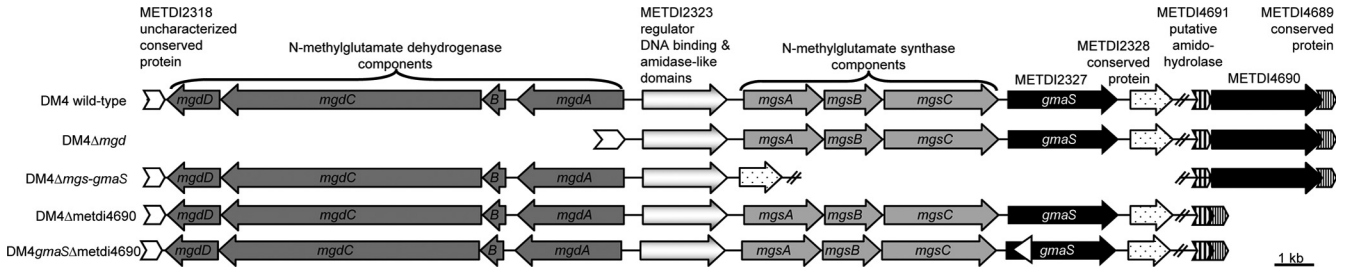

FIG 2 Gene organization of the NMG pathway-encoding genes in *M. extorquens* DM4 and constructed deletion mutants. The double slashes indicate distinct chromosome regions. The triangle shows the minitransposon insertion site and the orientation of the promoterless *gfp* gene relative to *gmaS* in mutant DM4*gmaS*Δmetdi4690.

complementary fragments, were PCR reamplified to yield a single PCR product in the second step. The resulting approximately 1-kb DNA fragment was digested with restriction enzymes to generate compatible cohesive ends and cloned into vector pCM433. The resulting plasmids, pME8282, pME8283, and pME8284 (for details, see Table S1), were electroporated into *E. coli* DH5α and then transferred to *M. extorquens* DM4 by triparental mating (12). Plasmid pME8282 was also introduced into the DM4*gmaS* mutant, in which *gmaS* was disrupted by minitransposon insertion to generate the double mutant DM4*gmaS*Δmetdi4690 strain. The mutants obtained (Fig. 2) were checked by sequencing the PCR-amplified genome deletion region.

Plasmid cloning of *gmaS* homologs. Homologs of *gmaS* were PCR amplified from wild-type DNA and cloned into the XbaI site of the P_{mxaF} promoter-based expression vector pCM80 (13), resulting in plasmids pME8280 and pME8285 harboring *gmaS* and the gene encoding METDI4690, respectively (for details, see Table S1). Insert sequences were verified to be of the wild type. Plasmids were transferred into *Methylobacterium* by conjugation as previously described (10).

Reverse transcription (RT)-PCR. RNA was extracted from cell pellets frozen at −80°C that were obtained by the centrifugation (5,000 rpm, 10 min, 4°C) of 50-ml liquid cultures of strain DM4 harvested at mid-exponential phase (OD_{600} = 0.2) as previously described (14). cDNA synthesized by using SuperScript III reverse transcriptase (Invitrogen) was used as the template for PCR amplification with iProof *Taq* polymerase (Bio-Rad) with the primer pairs listed in Table S1 in the supplemental material. RNA and cDNA concentrations were determined with Qubit2.0 (Invitrogen) according to the manufacturer's instructions.

RESULTS

Comparative analysis of the fully assembled genome sequences of the closely related *M. extorquens* strains DM4 and AM1 (15) surprisingly showed that strain DM4 lacked the well-studied *mau* genes for MMA utilization, although strain DM4 had been observed early on to grow with MMA (16). Reassessment of the ability of strains AM1 and DM4 to grow with MMA as the sole source of carbon and nitrogen then revealed clear differences in the growth rates of the two strains, with generation times of approximately 6 and 18 h, respectively (under the conditions used in this study). We therefore embarked on a search to identify and characterize the genes that allowed the growth of strain DM4 with MMA as the sole source of carbon and energy. This involved (i) the isolation and characterization of random minitransposon insertion mutants with a specific MMA-dependent phenotype when MMA was provided as the sole C source, (ii) site-directed mutagenesis and complementation assays to study the roles of specific genes or gene clusters in the utilization of MMA as a carbon or nitrogen source, and (iii) MMA-dependent gene expression by RT-PCR.

Isolation and characterization of MMA minitransposon insertion mutants. We started by screening a previously constructed library (10) of 6,054 mutants of strain DM4 capable of growing with 10 mM methanol and carrying random insertions of a minitransposon containing a promoterless *gfp* marker and a kanamycin resistance gene. Twenty-one mutants emitted MMA-dependent fluorescence or displayed impaired growth when tested on MMA, methanol, and a combination of the two compounds. Additional tests on M3 plates supplemented with a larger panel of one-carbon (dichloromethane, formate) and multicarbon (acetate, glycerol, pyruvate, betaine, and succinate) compounds were performed to identify mutants with specific MMA-dependent phenotypes. Seven mutants harboring a single minitransposon and displaying a specific MMA-dependent phenotype were obtained and classified into the following three groups: class 1, MMA-impaired growth with no detectable GFP fluorescence (three mutants); class 2, MMA-induced GFP fluorescence (two mutants); class 3, MMA-repressed GFP fluorescence (two mutants) (Table 1).

In four mutants, insertions colocalized within a 4-kb genomic region that encodes subunits of the NMG synthase (METDI2324, *mgsA*; METDI2325, *mgsB*; METDI2326, *mgsC*) and GMAS (METDI2327, *gmaS*) (Table 1; Fig. 2). The corresponding proteins displayed at least 66% amino acid sequence identity with characterized homologs in *M. silvestris* BL2 (1). Directly downstream of *gmaS* (Fig. 2), MMA-induced METDI2328 corresponds to a putative 169-residue-long protein with no homology to characterized proteins (mutant 07C5, Table 1). No GFP fluorescence emission was detected for mutants DM4*gmaS*, DM4*mgsA*, and DM4*mgsC* (Table 1, class 1), as expected, given that the minitransposon promoterless *gfp* gene was in reverse orientation with respect to the interrupted genes.

The other three mutants identified in this study showed MMA-dependent gene expression of the *gfp* minitransposon (Table 1), indicating differential expression of the genes at the minitransposon insertion site, i.e., genes *ureE*, *nuoG*, and those encoding the protein of unknown function METDI3639.

***gmaS* is essential for MMA utilization as the source of carbon but auxiliary for its utilization as the source of nitrogen.** Mutant DM4*gmaS* was unable to use MMA as the sole source of carbon and energy (Table 2). Minitransposon insertion into *gmaS* did not cause detrimental polar effects on the expression of adjacent genes, since wild-type *gmaS* provided in *trans* by plasmid pME8280 restored wild-type growth to mutant DM4*gmaS* (Table 3). We conclude that

TABLE 1 Characteristics of minitransposon mutants and reference strains with respect to growth on MMA

Interrupted locus[a]	Strain name	Insertion position[b]	Product of interrupted gene (gene)	GFP expression on MMA[c]	Growth on MMA[d]
Control strains					
None	DM4			Absent	Wild type
METDI2066	DM4*cycH*	1953319	Cytochrome *c*-type biogenesis protein (*cycH*)	Basal	Wild type
METDI4743	26F12	4639214	Conserved protein of unknown function	Basal	Wild type
Class 1, MMA-impaired growth					
METDI2324	DM4*mgsA*	2222651	NMG synthase component A (*mgsA*)	Absent	Altered
METDI2326	DM4*mgsC*	2224246	NMG synthase component C (*mgsC*)	Absent	Altered
METDI2327	DM4*gmaS*	2225098	GMA synthetase (*gmaS*)	Absent	Altered
Class 2, MMA-induced fluorescence					
METDI2328	07C5	2226639	Hypothetical protein	Induced	Wild type
METDI1773	DM4*ureE*	1646785	Putative urease accessory protein	Induced[e]	Wild type
Class 3, MMA-repressed fluorescence					
METDI1560	DM4*nuoG*	1424682	NADH:quinone oxidoreductase chain G (*nuoG*)	Downregulated	Altered
IG METDI3639-METDI3640	04B1	3582478		Downregulated	Wild type

[a] Mutants DM4*mgsA*, DM4*mgsC*, and DM4*gmaS* harbor the minitransposon *gfp* expression marker in reverse orientation with respect to the interrupted gene. In mutant 04B1, the minitransposon is located in an intergenic (IG) region with the *gfp* marker gene in the same orientation as METDI3639 downstream.
[b] Nucleotide position upstream of minitransposon I end.
[c] Fluorescence on solid medium with MMA. Induced, fluorescence observed only in the presence of MMA; enhanced, basal fluorescence enhanced in the presence of MMA; downregulated, fluorescence decreased in the presence of MMA alone or in combination with methanol; absent, no fluorescence.
[d] Wild-type or altered growth on solid M3 medium with MMA as the sole source of carbon and energy. All mutants displayed wild-type growth on methanol (20 mM), except mutant DM4*cycH*, which featured altered, weak growth on methanol.
[e] Urea did not induce GFP fluorescence.

gmaS is required for MMA utilization as a carbon source by *M. extorquens* DM4.

Regarding the assimilation of nitrogen, in contrast, when MMA was the only source of nitrogen in the presence of another carbon source, DM4*gmaS* grew about 10 times more slowly than the wild-type strain (generation times of 32.7 and 3.5 h, respectively; Table 2). However, no difference in growth ability between mutant DM4*gmaS* and the wild-type strain was observed when MMA was replaced with GMA, the product of MMA transformation by GMAS (Fig. 1). This suggested that GMA, which cannot be used by wild-type strain DM4 as the sole carbon source (Table 2, footnote *a*), can nevertheless enter the cell and serve as an alternative source of nitrogen, thereby alleviating the severe growth defect observed in the *gmaS* mutant.

The residual ability of the mutant DM4*gmaS* to grow with MMA as the sole nitrogen source suggested that other uncharacterized enzymes with GMAS activity may exist in *M. extorquens* DM4. We found another GMAS-like protein, METDI4690, which displayed 39% identity to GMAS (METDI2327) at the protein sequence level. To test whether METDI4690 could play a role in MMA utilization, additional mutants were generated by precisely deleting METDI4690 in both the wild-type and mutant DM4*gmaS* backgrounds, yielding the mutants DM4Δmetdi4690 and DM4*gmaS*Δmetdi4690, respectively. Mutant DM4Δmetdi4690

TABLE 2 Use of MMA or GMA as the sole carbon and/or nitrogen source for growth

Strain(s)	Avg generation time (h)[a] ± SD with:			
	MMA as C + N source	MMA as C source	MMA as N source	GMA as N source
DM4	18.3 ± 0.3	17.6 ± 0.6	3.5 ± 0.5	+[c]
07C5[b]	17.4 ± 0.7	17.7 ± 0.3	3.3 ± 0.1	ND[d]
DM4Δmetdi4690	18.4 ± 0.8	17.8 ± 0.5	3.3 ± 0.6	ND
DM4*gmaS*	No growth	No growth	32.7 ± 1.8	+[c]
DM4*gmaS*Δmetdi4690	No growth	No growth	38.5 ± 1.5	+[c]
DM4Δ*mgs-gmaS*, DM4*mgsC*	No growth	No growth	No growth	ND
DM4*mgsA*, DM4Δ*mgd*	No growth	No growth	No growth	No growth
DM4*nuoG*	No growth	No growth	3.3 ± 0.4	ND
DM4*ureE*	17.5 ± 0.4	17.4 ± 0.6	3.5 ± 0.2	ND

[a] Growth in M3N0 medium was tested (see Materials and Methods). When tested as a C source, MMA was provided at 20 mM. None of the strains grew with GMA as the sole C source (20 mM). When tested as N sources, MMA and GMA were provided at 1.5 and 0.75 mM, respectively. In controls, the N source was $(NH_4)_2SO_4$ at 1.5 mM and the C source was succinate at 5 mM. Growth with succinate and $(NH_4)_2SO_4$ resulted in a generation time of 2.5 ± 0.8 h for the wild-type strain and all of the other strains tested.
[b] Minitransposon insertion in METDI2328, the last CDS of the *mgsABC-gmaS*-METDI2328 operon.
[c] +, growth with flocculation prevented evaluation of generation times.
[d] ND, not determined.

TABLE 3 Characterization of growth of *M. extorquens* DM4 and mutants with MMA when provided with plasmid copies of GMAS homologs in *trans*

Interrupted[b] CDS(s) and plasmid[c]	Avg generation time (h) ± SD with MMA[a] provided as:		
	C + N source	C source	N source
None			
pCM80	18.9 ± 0.9	18.0 ± 0.4	4.4 ± 0.2
pME8280	16.2 ± 0.6	15.3 ± 0.3	3.3 ± 0.2
pME8285	17.5 ± 0.9	18.8 ± 1.6	3.5 ± 0.2
METDI2327			
pCM80	No growth	No growth	42.6 ± 1.2
pME8280	16.9 ± 0.7	15.9 ± 0.3	4.7 ± 0.3
pME8285	20.2 ± 0.8	16.5 ± 1.3	4.0 ± 0.3
METDI2327, METDI4690			
pCM80	No growth	No growth	41.7 ± 1.2
pME8285	21.5 ± 0.5	19.2 ± 1.5	4.0 ± 0.1

[a] MMA was added as the C or N source at 20 or 1.5 mM, respectively. In controls, succinate at 5 mM and $(NH_4)_2SO_4$ at 1.5 mM were provided as C and N sources, respectively. Similar growth of all strains on succinate (5 mM) and $(NH_4)_2SO_4$ (1.5 mM) was observed (generation time, 3.4 ± 0.3 h).
[b] Interruption of METDI4690 alone conferred no phenotype with MMA as the C or N source (Table 2, mutant DM4Δmetdi4690).
[c] pCM80, empty expression vector; pME8280, pCM80 for *gmaS* expression of METDI2327; pCM8285, pCM80 for expression of METDI4690.

itself grew identically to the wild type under all of the conditions tested (Table 2). Only in mutant DM4*gmaS*Δmetdi4690 lacking both *gmaS* homologs, when MMA was provided as the sole nitrogen source, did the lack of METDI4690 have a detectable effect on growth (generation time of 38.5 h for DM4*gmaS*Δmetdi4690 versus 32.7 h for DM4*gmaS*, Table 2). Thus, MMA utilization as the sole nitrogen source involves both *gmaS* homologs, although METDI2327 (GMAS) plays a major role compared to that of METDI4690 in the wild-type context (Table 3).

***mgsABC* and *mgdABCD* are essential genes for the growth of *M. extorquens* DM4 with MMA as the source of carbon and nitrogen.** NMG synthase is a poorly characterized enzyme that is homologous to glutamate synthase (17), which, by analogy, may have the capacity to transform both MMA and GMA obtained from MMA by GMAS with the concomitant release of ammonia (Fig. 1). Genes homologous to *mgsABC* encoding NMG synthase were found to be essential for growth with MMA in strain DM4, as transposon insertion mutants DM4*mgsA* and DM4*mgsC* were unable to grow with MMA as either a carbon or a nitrogen source (Table 2). Similarly, mutant DM4*mgsA* was also unable to grow with GMA as a nitrogen source in the presence of another carbon source.

The following reaction in the NMG pathway is catalyzed by NMG dehydrogenase encoded by the genes *mgdABCD* and funnels the methyl group carbon of NMG into methylenetetrahydrofolate (Fig. 1). On the basis of sequence similarity, as well as experimental data, the *mgdABCD* genes constitute the last specific step of the NMG pathway (Fig. 1) (6). Very similar genes are known to encode sarcosine (*N*-methylglycine) oxidase and are found in many bacteria, including *Methylobacterium* strains, which contain at least two sets of homologs. Homologs of the *mgdABCD* genes, sharing 73, 27, 55, and 21% amino acid sequence identity with characterized components A, B, C, and D of the NMG dehydrogenase of *M. silvestris* BL2 (1), were found upstream of the *mgsABC-gmaS* region in strain DM4 (Fig. 2). Somewhat surprisingly, no mutants with minitransposon insertions in the *mgdABCD* genes were detected. Therefore, we constructed a mutant with all of the *mgdABCD* genes deleted in order to assess if the *mgd* cluster plays a role in MMA utilization by *M. extorquens* DM4. The resulting DM4Δ*mgd* mutant was unable to grow in the presence of MMA as the sole source of carbon and energy and also as the sole source of nitrogen in the presence of another carbon source for growth (Table 2). In conclusion, both the *mgs* and *mgd* gene clusters are essential for MMA utilization as a carbon, energy, and nitrogen source by *M. extorquens* DM4.

The *nuoG* gene is essential for strain DM4 growth with MMA as the carbon and energy source. Analysis of mutant DM4*nuoG* showed that disruption of *nuoG* prevented the growth of strain DM4 with MMA as the sole carbon source but not as the sole nitrogen source when growing with succinate as the carbon source (Table 2). The gene *nuoG* is 1 of the 14 genes of the *nuo* cluster that encodes NADH:ubiquinone oxidoreductase, a respiratory complex 1 enzyme that catalyzes the transfer of electrons from NADH to the quinone pool, coupled with translocation of protons across the membrane. Disruption of individual *nuo* genes impairs respiratory complex 1 in *E. coli* (18). The inability of mutant DM4*nuoG* to grow with MMA as the carbon and energy source suggests that NuoG may be involved in accepting electrons from the oxidation of MMA by the NMG pathway in strain DM4. In contrast, mutant DM4*cycH*, in which the disrupted gene is involved in cytochrome *c* biogenesis (19), was able to grow with MMA by the NMG pathway but not with methanol. Thus, *M. extorquens* DM4 appears to require the cytochrome *c* electron transfer system for oxidation of the C_1 alcohol methanol but not of the corresponding C_1 amine MMA.

MMA-dependent gene expression. By using RT-PCR amplifications targeting intergenic regions in the vicinity of the genes *mgd*, *mgs*, and *gmaS*, the expected operonic structure of the divergent *mgdABCD* and *mgsABC-gmaS*-METDI2328 gene clusters was confirmed (Fig. 3A). MMA-dependent regulation was assessed with cultures of strain DM4 grown with MMA, methanol, or succinate as the sole carbon source. The genes *mgdC*, *mgsA*, and *gmaS* and the MMA-associated *ureE* homolog displayed MMA-dependent upregulation, as shown by RT-PCR with intragenic gene primer pairs (Fig. 3B). Conversely, *nuoG* and the putative transcriptional regulator METDI2323 located between the two operons were downregulated by MMA (Fig. 3B). Taken together, the data obtained showed that the genome region encoding the NMG pathway (Fig. 2) harbors two MMA-induced, divergently transcribed operons (*mgdABCD* and *mgsABC-gmaS*-METDI2328) separated by a putative regulator gene whose expression was downregulated by MMA or by a metabolite generated in the course of MMA utilization. In contrast, for genes encoding the GMAS homolog METDI4690 and two proteins of unknown functions, METDI2318 and METDI3639 (located downstream of the insertion site in minitransposon mutant 04B1; Table 1), constitutive low-level expression irrespectively of the presence of MMA in the medium was found (Fig. 3B, right panel).

Effect of plasmid-driven expression of *gmaS* homologs. The constitutive low-level expression of METDI4690 suggested by RT-PCR experiments (Fig. 3B) may explain why deletion of the gene that encodes it had little effect on the ability of strain DM4 to utilize MMA (Table 2). To test this hypothesis, plasmid pME8285

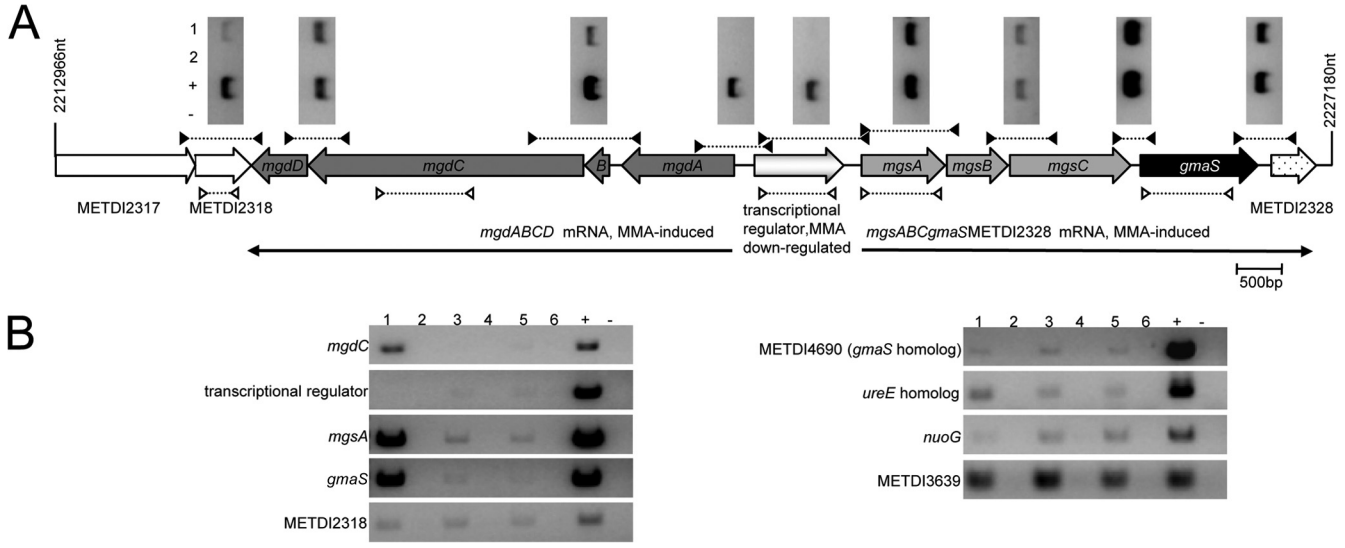

FIG 3 Transcription studies of MMA-dependent gene expression in *M. extorquens* DM4 by PCR on reverse transcriptase-produced total cDNA. RNA was isolated from cultures grown with MMA (lines 1 and 2) or with $(NH_4)_2SO_4$ as a nitrogen source with either methanol (lines 3 and 4) or succinate (lines 5 and 6) as a carbon source and reverse transcribed, and the cDNA was quantified before PCR amplification. Controls were performed for each primer pair with *M. extorquens* DM4 genomic DNA (1.5 ng) (lane +), water (lane −), and samples from which RT was omitted prior to PCR amplification (lanes 2, 4, and 6). (A) PCR amplification of cDNA (4 ng) was performed with primer pairs spanning intergenic regions (black triangles). Thick arrows denote experimentally confirmed operonic structures. (B) PCR amplification from cDNA of individual genes (primer pairs are indicated by white triangles) of the NMG pathway (left) and of other genes investigated in this study (right), starting from 8 ng total cDNA, except for the transcriptional regulator (16 ng cDNA).

harboring cloned METDI4690 under the control of promoters P_{tac} and P_{mxaF} of vector pCM80, which favor strong constitutive expression of downstream genes in pCM plasmids (9, 15, 20), was introduced into wild-type and *gmaS* mutant strain DM4. No significant difference in growth with MMA as the sole source of carbon and nitrogen was observed in the wild-type strain harboring plasmid pME8285 or the empty expression vector pCM80 (Table 3). Similarly, providing GMAS (METDI2327) in *trans* from plasmid pME8280 allowed only slightly faster growth of the wild-type strain with MMA as the source of carbon or nitrogen (Table 3) and fully complemented the growth defect of mutant DM4*gmaS* (Table 2). However, providing METDI4690 in *trans* on pME8285 restored the ability of mutants lacking one or both *gmaS* homologs to use MMA as a source of carbon and nitrogen for growth (Table 3). Thus, METDI4690 represents a bona fide functional GMAS that may, however, be too weakly expressed from its native chromosomal promoter to sustain the utilization of MMA as the sole carbon source by *M. extorquens* DM4. We concluded that both the gene encoding METDI2327 and METDI4690 *gmaS* homologs express functional GMAS enzymes but that their expression is the limiting factor for optimal MMA utilization under the conditions tested.

Phylogenetic analysis of *gmaS* homologs and comparison of gene clusters. Proteins encoded by *gmaS* homologs belong to the larger protein family of glutamine synthetases, not all of which are able to transform MMA (1, 21). The sequences of *M. extorquens* DM4 *gmaS* homologs METDI2327 and METDI4690 were compared to those of strain DM4 homologs of the three known families of glutamine synthetases (METDI2616, METDI3157, and METDI3158) to experimentally characterized GMAS enzymes and to representative homologs of other prokaryotes and eukaryotes, as well as to sequences closely related to METDI4690 from representative sequenced genomes (at least 44% identity at the protein level, lengths of 444 to 472 amino acids; see Table S2 in the supplemental material). The resulting phylogenetic tree (Fig. 4) confirms the previously documented four types of glutamine synthetase homologs (1, 22), i.e., the three glutamine synthetase types and the group containing all of the GMAS enzymes experimentally characterized so far. DM4 homologs METDI2616, METDI3157, and METDI3158 can be assigned to glutamine synthetase types I and II, but the MMA-associated *gmaS* homologs METDI2327 and METDI4690 investigated here clearly cluster within the GMAS group. Notably, METDI4690-like sequences (framed in Fig. 4) are found mainly in members of the class *Proteobacteria* (see Table S2) but are only loosely associated with methylotrophic metabolism, in contrast to the closely related homologs of the experimentally characterized GMAS and METDI2327 of strain DM4.

However, METDI237- and METDI4690-like *gmaS* homologs could be distinguished by taking their genetic organization context into account (Fig. 5). Previously experimentally characterized, bona fide *gmaS* genes colocate with genes encoding enzymes of the NMG pathway of MMA oxidation, especially with *mgs* genes (1, 6) (Fig. 5B). In contrast, homologs of METDI4690, shown here to restore the growth of strain DM4 with MMA as a carbon and nitrogen source, in mutants impaired in the NMG pathway (Table 3) were often associated with a conserved gene encoding an uncharacterized amidohydrolase (conserved domain COG1402) described as potentially acting on nonpeptidic C-N bonds (Fig. 5B). Genes encoding components of an ABC-type transporter and proteins involved in urea-related metabolism were also often found in close proximity (Fig. 5B). Thus, genes encoding GMAS homologs closely similar to METDI4690 appear to be associated with a conserved uncharacterized gene cluster involved in nitrogen metabolism.

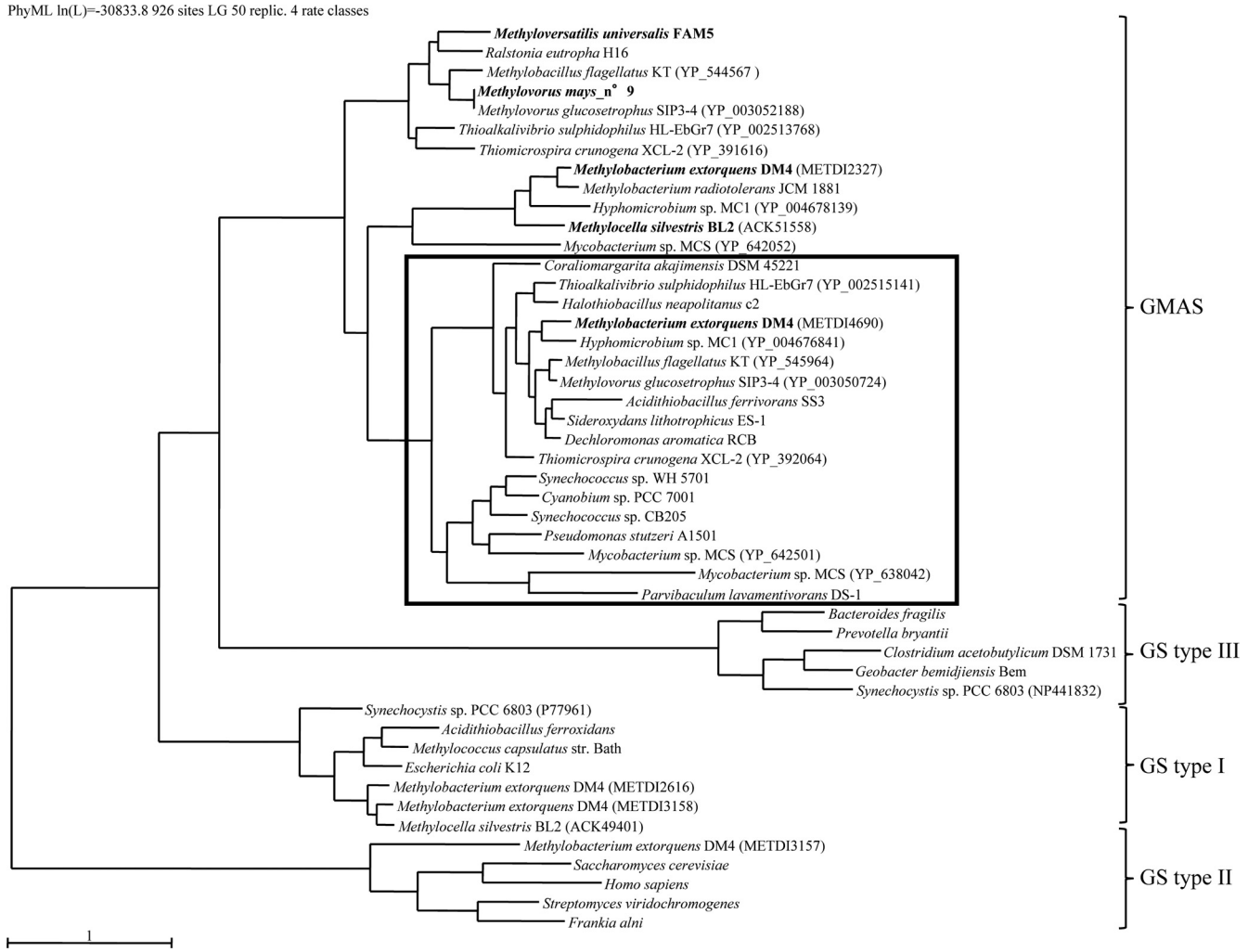

FIG 4 Phylogenetic tree representation of protein sequences encoded by homologs of *gmaS* and representatives of the three known types of glutamine synthetase (GS). The tree was constructed by using PhyML. Organisms with experimentally characterized GMAS are in bold. Close homologs of METDI4690 are framed. For nucleotide sequence accession numbers, protein sequence lengths, and percentages of identity with *M. extorquens* GMAS and METDI4690, see Table S2 in the supplemental material. The accession number of each bacterium in which more than one *gmaS* homolog was found is shown in parentheses.

DISCUSSION

We demonstrated in this work that the two closely adjacent, divergent operons, *mgdABCD* and *mgsABC-gmaS*-METDI2328, involved in the NMG pathway are induced by MMA and allow *M. extorquens* DM4 to use MMA as a sole carbon, nitrogen, and energy source. The two operons are separated by only a putative regulator gene (the gene for METDI2323) whose expression is downregulated by MMA (Fig. 3) and which belongs to the AraC family of transcriptional regulators with a C-terminal DNA-binding helix-turn-helix domain (PROSITE PS01124). In addition, METDI2323 features an N-terminal class I glutamine amidotransferase-like domain that may serve to sense a chemical effector such as MMA or another compound associated with MMA metabolism. The last gene of the cluster cotranscribed with *gmaS* encodes the 169-residue-long protein METDI2328 of unknown function with close homologs so far found only in *Methylobacterium* genomes. This gene is not essential for MMA metabolism, since its disruption had no effect on the ability of strain DM4 to grow with MMA under any of the conditions tested (Fig. 3; Table 1, mutant 07C5). Nevertheless, its expression is induced by MMA, as is that of a *ureE* homolog for a urease accessory protein (23). The transcription of urease genes can be regulated by nitrogen availability (24), and we speculate that this MMA-induced urease-like operon may contribute in some way to MMA-associated nitrogen assimilation in strain DM4. Nevertheless and as for METDI2328, disruption of *ureE* by minitransposon insertion in mutant DM4*ureE* is not associated with any detected growth defect (Table 1). The presence of two homologs of the seven-gene urease operon *ureEFABCGD* in strain DM4 may explain this observation.

Unlike *M. extorquens* strains DM4, PA1, and BJ001 (8, 25), *M. extorquens* strains such as AM1 and CM4 contain *mau* genes and grow much faster with MMA as the sole carbon, nitrogen, and energy source (approximately 3-fold growth rate difference; C. Gruffaz and F. Bringel, unpublished data). Moreover, *M. extorquens* DM4 grew less efficiently with MMA than with methanol (generation times of 18.3 and 3.4 h, respectively; Table 2) (10). It is possible that energy production by the oxidation of one-carbon compounds involves different pathways of electron transfer for

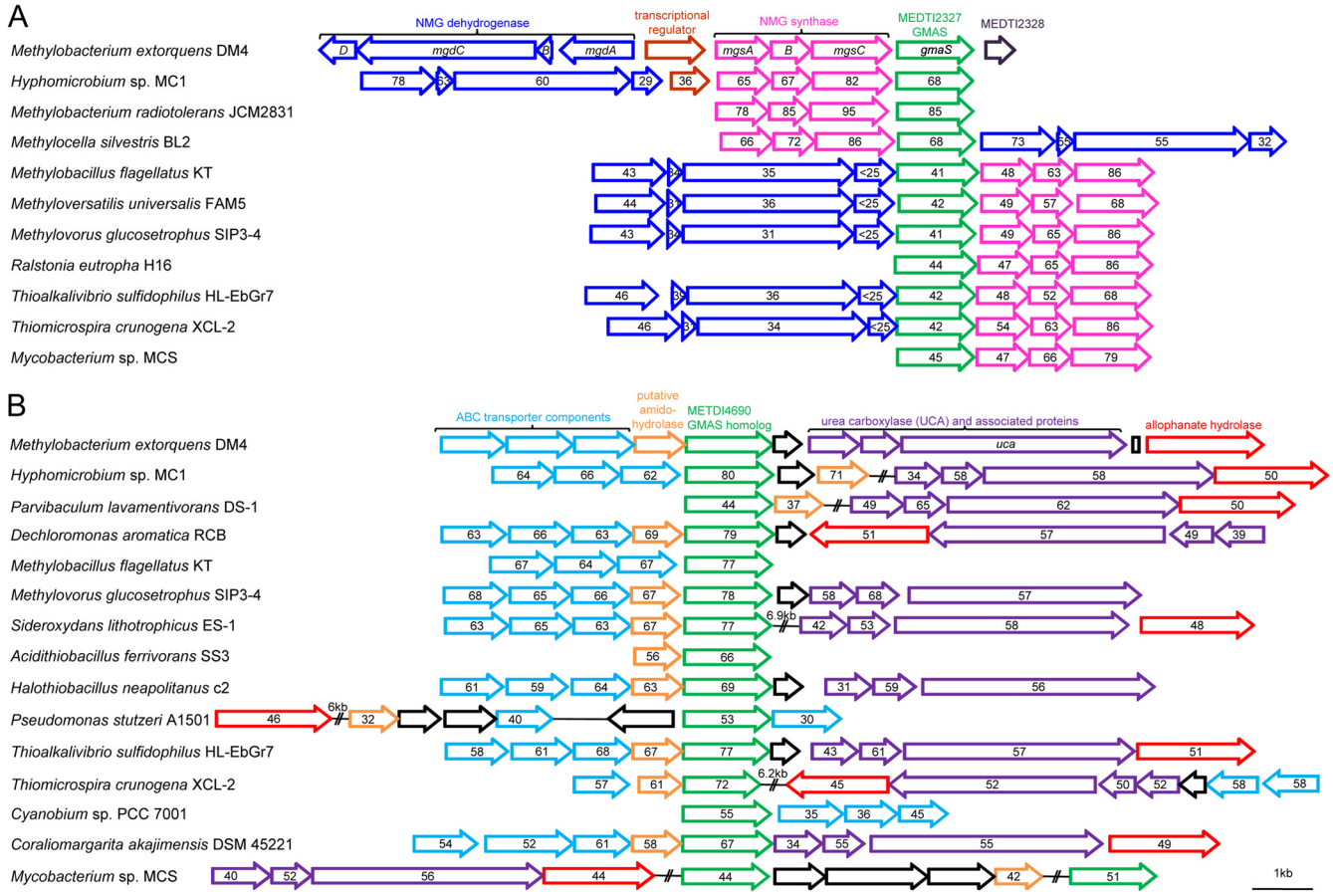

FIG 5 Gene organization and conservation of *gmaS* homolog-containing genomic regions for sequences closely related to METDI2327 (A) and METI4690 (B), respectively. Gene orientation and linkage are indicated with arrows, and double slashes indicate a distance of at least 10 kb between the genome regions considered. UCA-associated proteins 1 and 2 are encoded by a tandem of homologous, uncharacterized genes with conserved domains TIGR03424 and TIGR03425, respectively. *Alphaproteobacteria* class members (complete genome accession numbers are in parentheses): *M. extorquens* DM4 (NC_012988), *Hyphomicrobium* sp. strain MC1 (NC_015717), *Methylobacterium radiotolerans* JCM2831 (NC_010505), *M. silvestris* BL2 (NC_011666), and *Parvibaculum lavamentivorans* DS-1 (NC_009719). *Betaproteobacteria* class members: *Dechloromonas aromatica* RCB (NC_007298), *Methylobacillus flagellatus* KT (NC_007947), *M. universalis* FAM5 (NZ_AFHG00000000), *Methylovorus glucosetrophus* SIP3-4 (NC_012969), *Sideroxydans lithotrophicus* ES-1 (NC_013959), and *Ralstonia eutropha* H16 (NC_008314). *Gammaproteobacteria* class members: *Acidithiobacillus ferrivorans* SS3 (NC_015942), *Halothiobacillus neapolitanus* c2 (NC_013422), *Pseudomonas stutzeri* A1501 (NC_009434), *Thioalkalivibrio sulfidophilus* HL-EbGr7 (NC_011901), and *Thiomicrospira crunogena* XCL-2 (NC_007520). *Cyanobacteria* class member: *Cyanobium* sp. strain PCC 7001 (NZ_DS990556). *Verrucomicrobia* class member: *Coraliomargarita akajimensis* DSM 45221 (NC_014008). *Actinobacteria* class member: *Mycobacterium* sp. strain MCS (NC_008147).

MMA oxidation (by the NMG pathway) and for methanol (by methanol dehydrogenase), as suggested by the different growth phenotypes of mutants DM4*nuoG* and DM4*cycH* (Tables 1 and 2). Still, the genetic organization of NMG pathway genes in *M. extorquens* DM4 is strongly conserved within the sequenced genomes of *M. extorquens* (8, 25), suggesting that possession of this gene set would allow all *Methylobacterium* strains to grow with MMA. That is not the case, since inactivation of *mau* genes for MADH in the closely related strain AM1 completely abolished its ability to grow on MMA as the sole C source (4). Nevertheless, NMG dehydrogenase activity was detected in *M. extorquens* AM1 (4, 19), suggesting that other factors beyond the mere possession of the required genes are required for the growth of *Methylobacterium* strains with MMA by the NMG pathway.

The observed growth phenotypes of NMG pathway mutants have shed some light on the still somewhat elusive roles of GMAS and NMG synthase in the growth of strain DM4 with MMA (Table 3). The fact that a mutant with impaired *mgsA* is unable to use MMA or GMA as either a carbon or a nitrogen source for growth represents the most clear-cut result obtained in this work (Table 2). It demonstrates the absolutely essential role of NMG synthase in the NMG pathway (Fig. 1), most likely involving the same glutamate–α-ketoglutarate redox couple as in the homologous enzyme glutamate synthase (17) for condensation of amines to α-ketoglutarate with concomitant release of ammonia, which can then be used as a nitrogen source for growth. We have also demonstrated that in the absence of a functional *gmaS* gene (mutant DM4*gmaS*), *M. extorquens* DM4 lacks the ability to utilize MMA as the sole carbon and energy source, suggesting that GMA is an obligate intermediate of MMA oxidation by the NMG pathway when MMA is the sole source of carbon and energy (Fig. 1). Nevertheless, GMA as the sole source of carbon and energy did not sustain the growth of wild-type strain DM4, unlike when it was provided as the sole nitrogen source in the presence of another carbon source. It remains to be tested whether GMA is transported efficiently enough within the cell to fulfill the higher car-

bon rather than nitrogen assimilation requirements for cell growth. When considering published studies, the question of whether GMA is an obligate intermediate of the NMG pathway (Fig. 1) is still debated, given the contradictory experimental evidence obtained so far for the role of GMAS in *M. universalis* FAM5 and *M. sylvestris* BL2. In *M. silvestris* BL2, *gmaS* is essential for MMA utilization as a C and energy source (1), whereas this gene is unexpectedly dispensable in *M. universalis* FAM5 (6). Intriguingly, *M. universalis* FAM5 and *M. silvestris* BL2 are bacteria that contain only one *gmaS* homolog (see Table S2 in the supplemental material), in contrast to all of the strains of *M. extorquens* sequenced so far (8, 25), which contain two *gmaS* homologs (Fig. 5). In *M. extorquens* DM4, GMAS (METDI2327) was found to be required for MMA utilization as a carbon source. This suggests that the direct reaction of MMA with glutamate catalyzed by NMG synthase is not sufficiently efficient to bypass mutation in *gmaS* with respect to carbon requirements for growth. In other words, the indirect pathway through *gmaS* and GMA as an intermediate may be a more efficient way to metabolize carbon from MMA. Deletion of the second *gmaS* homolog, METDI4690, further decreased the residual capacity of the DM4*gmaS* mutant to grow with MMA as a nitrogen source, and deletion of the entire gene cluster encoding NMG synthase and GMAS completely abolished the ability of strain DM4 to utilize MMA as a nitrogen source for growth (Table 3). This demonstrates that no enzymatic systems beyond the two GMAS homologs exist in strain DM4 to extract nitrogen from MMA in a growth-conducive fashion. Most notably, complementation with either GMAS METDI2327 or METDI4690 on a multicopy plasmid in both the DM4*gmaS* and DM4*gmaS*Δmetdi4690 backgrounds allowed the utilization of MMA as the sole source of carbon and nitrogen for growth (Table 3) and conclusively demonstrated that both GMAS homologs of strain DM4 were bona fide GMAS proteins. In addition, plasmid expression of either GMAS homolog was found to significantly improve the ability of *M. extorquens* DM4 to utilize MMA as the sole source of carbon, energy, and nitrogen for growth (Table 3). Thus, expression of *gmaS* homologs may represent a major bottleneck in the ability of methylotrophic bacteria to grow with methylated amines. The distinct expression levels of the two *gmaS* homologs in wild-type strain DM4 (Fig. 3) suggest that they are involved in different metabolic pathways associated with the transformation of compounds containing amino groups and that MMA-induced METDI2327 plays a predominant role in MMA oxidation. METDI4690, in contrast, is expressed at low constitutive levels and lies immediately adjacent to genes encoding components of the urea carboxylase (UCA)/allophanate hydrolase pathway, as in many bacteria known to use urea as the sole nitrogen source (26) (Fig. 5). The *uca* gene adjacent to METDI4690 may therefore be involved in the interconversion of methylated amides and amines for nitrogen metabolism. Indeed, in *Oleomonas sagaranensis*, a member of the class *Alphaproteobacteria*, ammonia is produced by UCA not only from urea degradation but also from acetamide and formamide (26). Thus, *gmaS* homologs, which are widely distributed in bacterial genomes, may contribute to the microbial metabolism of a large variety of amides and amines found in the environment.

A final point of the present study is that the NMG pathway downstream of NMG involves aspects that are not yet understood. Unexpectedly, mutant DM4Δ*mgd* was unable to utilize MMA or GMA as a nitrogen source when succinate was supplied as a carbon source (Table 2), even though ammonium as a growth-supporting source of nitrogen is not generated directly through NMG dehydrogenase activity (Fig. 1). One of several possible explanations for the phenotype of mutant DM4Δ*mgd* is that lack of NMG dehydrogenase somehow inactivates NMG synthase. This hypothesis remains to be tested, as well as the possible key role of glutamate as an intermediate, substrate, and/or product of all three steps of the NMG pathway.

ACKNOWLEDGMENTS

This work was supported by the Région Alsace for the Ph.D. grant of E.E.L.M.

Help in mutant library screening from S. El Hassoun and constructive discussions with S. Vuilleumier are acknowledged.

REFERENCES

1. **Chen Y, Scanlan J, Song L, Crombie A, Rahman MT, Schäfer H, Murrell JC.** 2010. γ-Glutamylmethylamide is an essential intermediate in the metabolism of methylamine by *Methylocella silvestris*. Appl. Environ. Microbiol. **76:**4530–4537. http://dx.doi.org/10.1128/AEM.00739-10.
2. **Anthony C.** 1982. The biochemistry of methylotrophs. Academic Press, Inc., New York, NY.
3. **Bicknell B, Owens JD.** 1980. Utilization of methyl amines as nitrogen sources by non-methylotrophs. J. Gen. Microbiol. **117:**89–96.
4. **Chistoserdov AY, Chistoserdova LV, McIntire WS, Lidstrom ME.** 1994. Genetic organization of the *mau* gene cluster in *Methylobacterium extorquens* AM1: complete nucleotide sequence and generation and characteristics of *mau* mutants. J. Bacteriol. **176:**4052–4065.
5. **Chistoserdova L.** 2011. Modularity of methylotrophy, revisited. Environ. Microbiol. **13:**2603–2622. http://dx.doi.org/10.1111/j.1462-2920.2011.02464.x.
6. **Latypova E, Yang S, Wang Y-S, Wang T, Chavkin TA, Hackett M, Schäfer H, Kalyuzhnaya MG.** 2010. Genetics of the glutamate-mediated methylamine utilization pathway in the facultative methylotrophic beta-proteobacterium *Methyloversatilis universalis* FAM5. Mol. Microbiol. **75:**426–439. http://dx.doi.org/10.1111/j.1365-2958.2009.06989.x.
7. **Chen Y, McAleer KL, Murrell JC.** 2010. Monomethylamine as a nitrogen source for a nonmethylotrophic bacterium, *Agrobacterium tumefaciens*. Appl. Environ. Microbiol. **76:**4102–4104. http://dx.doi.org/10.1128/AEM.00469-10.
8. **Vuilleumier S, Chistoserdova L, Lee M-C, Bringel F, Lajus A, Zhou Y, Gourion B, Barbe V, Chang J, Cruveiller S, Dossat C, Gillett W, Gruffaz C, Haugen E, Hourcade E, Levy R, Mangenot S, Muller E, Nadalig T, Pagni M, Penny C, Peyraud R, Robinson DG, Roche D, Rouy Z, Saenampechek C, Salvignol G, Vallenet D, Wu Z, Marx CJ, Vorholt JA, Olson MV, Kaul R, Weissenbach J, Médigue C, Lidstrom ME.** 2009. *Methylobacterium* genome sequences: a reference blueprint to investigate microbial metabolism of C_1 compounds from natural and industrial sources. PLoS One **4:**e5584. http://dx.doi.org/10.1371/journal.pone.0005584.
9. **Roselli S, Nadalig T, Vuilleumier S, Bringel F.** 2013. The 380 kb pCMU01 plasmid encodes chloromethane utilization genes and redundant genes for vitamin B_{12}- and tetrahydrofolate-dependent chloromethane metabolism in *Methylobacterium extorquens* CM4: a proteomic and bioinformatics study. PLoS One **8:**e56598. http://dx.doi.org/10.1371/journal.pone.0056598.
10. **Muller EEL, Hourcade E, Louhichi-Jelail Y, Hammann P, Vuilleumier S, Bringel F.** 2011. Functional genomics of dichloromethane utilization in *Methylobacterium extorquens* DM4. Environ. Microbiol. **13:**2518–2535. http://dx.doi.org/10.1111/j.1462-2920.2011.02524.x.
11. **Zhang Z, Schwartz S, Wagner L, Miller W.** 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. **7:**203–214. http://dx.doi.org/10.1089/10665270050081478.
12. **Marx CJ.** 2008. Development of a broad-host-range *sacB*-based vector for unmarked allelic exchange. BMC Res. Notes **1:**1. http://dx.doi.org/10.1186/1756-0500-1-1.
13. **Marx CJ, Lidstrom ME.** 2001. Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. Microbiology **147:**2065–2075.
14. **Marchal M, Briandet R, Halter D, Koechler S, DuBow MS, Lett M-C,**

Bertin PN. 2011. Subinhibitory arsenite concentrations lead to population dispersal in *Thiomonas* sp. PLoS One **6:**e23181. http://dx.doi.org/10.1371/journal.pone.0023181.
15. **Kayser MF, Ucurum Z, Vuilleumier S.** 2002. Dichloromethane metabolism and C_1 utilization genes in *Methylobacterium* strains. Microbiology **148:**1915–1922.
16. **Gälli R.** 1986. Optimierung des mikrobiellen Abbaus von Dichlormethan in einem Wirbelschicht-Bioreaktor. Eidgenössische Technische Hochschule, Zürich, Switzerland.
17. **Vanoni MA, Curti B.** 2008. Structure-function studies of glutamate synthases: a class of self-regulated iron-sulfur flavoenzymes essential for nitrogen assimilation. IUBMB Life **60:**287–300. http://dx.doi.org/10.1002/iub.52.
18. **Erhardt H, Steimle S, Muders V, Pohl T, Walter J, Friedrich T.** 2012. Disruption of individual *nuo*-genes leads to the formation of partially assembled NADH:ubiquinone oxidoreductase (complex I) in *Escherichia coli*. Biochim. Biophys. Acta **1817:**863–871. http://dx.doi.org/10.1016/j.bbabio.2011.10.008.
19. **Page MD, Ferguson SJ.** 1995. Cloning and sequence analysis of *cycH* gene from *Paracoccus denitrificans*: the *cycH* gene product is required for assembly of all *c*-type cytochromes, including cytochrome c_1. Mol. Microbiol. **15:**307–318.
20. **Marx CJ, Lidstrom ME.** 2004. Development of an insertional expression vector system for *Methylobacterium extorquens* AM1 and generation of null mutants lacking *mtdA* and/or *fch*. Microbiology **150:**9–19. http://dx.doi.org/10.1099/mic.0.26587-0.
21. **Brown JR, Masuchi Y, Robb FT, Doolittle WF.** 1994. Evolutionary relationships of bacterial and archaeal glutamine synthetase genes. J. Mol. Evol. **38:**566–576.
22. **Merrick MJ, Edwards RA.** 1995. Nitrogen control in bacteria. Microbiol. Rev. **59:**604–622.
23. **Farrugia MA, Macomber L, Hausinger RP.** 2013. Biosynthesis of the urease metallocenter. J. Biol. Chem. **288:**13178–13185. http://dx.doi.org/10.1074/jbc.R112.446526.
24. **Mobley HL, Hausinger RP.** 1989. Microbial ureases: significance, regulation, and molecular characterization. Microbiol. Rev. **53:**85–108.
25. **Marx CJ, Bringel F, Chistoserdova L, Moulin L, Farhan Ul Haque M, Fleischman DE, Gruffaz C, Jourand P, Knief C, Lee M-C, Muller EEL, Nadalig T, Peyraud R, Roselli S, Russ L, Goodwin LA, Ivanova N, Kyrpides N, Lajus A, Land ML, Médigue C, Mikhailova N, Nolan M, Woyke T, Stolyar S, Vorholt JA, Vuilleumier S.** 2012. Complete genome sequences of six strains of the genus *Methylobacterium*. J. Bacteriol. **194:**4746–4748. http://dx.doi.org/10.1128/JB.01009-12.
26. **Kanamori T, Kanou N, Atomi H, Imanaka T.** 2004. Enzymatic characterization of a prokaryotic urea carboxylase. J. Bacteriol. **186:**2532–2539. http://dx.doi.org/10.1128/JB.186.9.2532-2539.2004.
27. **Martinez-Gomez NC, Nguyen S, Lidstrom ME.** 2013. Elucidation of the role of the methylene-tetrahydromethanopterin dehydrogenase MtdA in the tetrahydromethanopterin-dependent oxidation pathway in *Methylobacterium extorquens* AM1. J. Bacteriol. **195:**2359–2367. http://dx.doi.org/10.1128/JB.00029-13.