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γ-Glutamylmethylamide Is an Essential Intermediate in the Metabolism of Methylamine by *Methylocella silvestris*▽

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***Methylocella silvestris* BL2, a facultative methane utilizer, can grow on monomethylamine (MMA) as a sole carbon and nitrogen source. No activity of MMA dehydrogenase was detectable. Instead, this bacterium utilizes a methylated amino acid pathway (γ-glutamylmethylamide [GMA] and *N*-methylglutamate [NMG]) for MMA metabolism. The activities of the two key enzymes in this pathway, GMA synthetase and NMG dehydrogenase, were found when the bacterium was grown on MMA. GMA was detected by high-performance liquid chromatography–mass spectrometry only when the bacterium was grown on MMA but not when it was grown on methanol. Proteomic analysis of soluble and membrane fractions of the proteome from MMA- and methanol-grown cultures revealed that an eight-gene cluster (Msil2632 to Msil2639) was induced by MMA and cotranscribed as an operon, as shown by reverse transcription-PCR. GMA-dissimilating enzyme activity was also detected when it was grown on MMA. Formaldehyde and ammonium production from GMA was dependent on glutamate but not on α-ketoglutarate. Marker exchange mutagenesis of a putative GMAS gene homologue (*gmas*, Msil2635) within this eight-gene cluster, with a kanamycin gene cassette, abolished growth of *M. silvestris* on MMA as either a sole carbon or a sole nitrogen source. Overall, our results suggest that *gmas* is essential in MMA metabolism by *M. silvestris*.**

Monomethylamine (MMA) is ubiquitous in the environment. For example, putrefaction of proteins (14a, 17) and degradation of many nitrogen-containing pesticides and herbicides can release MMA (5, 16b, 18). In the marine environment, MMA is released from the degradation of quaternary amines, such as betaine, carnitine, choline, and trimethylamine *N*-oxide, which are used as osmolytes by many marine organisms (3, 6). Once released, MMA can be used by some microorganisms as a sole carbon and nitrogen source through different pathways. Methanogenic archaea, such as *Methanosarcina* and *Methanomicrobium*, can use MMA anaerobically as a substrate to produce methane via a methyltransferase system (28). Gram-positive bacteria, such as *Arthrobacter*, metabolize MMA aerobically via an oxidase, which breaks down MMA into formaldehyde and ammonium (39). Gram-negative bacteria such as *Methylobacterium extorquens* and *Paracoccus denitrificans* utilize MMA dehydrogenase, a multisubunit enzyme that generates formaldehyde and ammonium from MMA aerobically (9, 16). Many other Gram-negative bacteria, such as *Aminobacter aminovorans* (previously known as strain MA and strain MS), can use MMA as a sole carbon and nitrogen source aerobically; however, they lack MMA dehydrogenase. It has been shown that in these microorganisms, two unusual amino acids, γ-glutamylmethylamide (GMA) and *N*-methylglutamate (NMG), are involved in MMA metabolism (1, 23, 33). In strain MA, an enzyme proposed as "NMG synthase" ("NMGS") converted MMA to NMG, which was subsequently oxidized to formaldehyde, regenerating glutamate (Glu) by a membrane-bounded particulate, NMG dehydrogenase (NMGDH) (33). The reactions carried out by these enzymes are summarized below. In strain MS, GMA was found to be a key metabolite in MMA metabolism (23). The synthesis of GMA was proposed to be carried out by a glutamine synthetase-like protein, and the reaction was dependent on ATP and Mg^{2+}. However, the fate of GMA in such a GMA-dependent MMA pathway is not clear.

$$\text{"NMG synthase" ("NMGS"): Glu + MMA} \rightarrow \text{NMG + } NH_4^+$$

$$\text{NMG dehydrogenase (NMGDH): NMG} \rightarrow \text{HCHO + Glu}$$

$$\text{GMA synthetase (GMAS): Glu + MMA + ATP} \rightarrow \text{GMA + ADP + } P_i$$

Although enzymes in these methylated amine-mediated pathways have been purified earlier, such as "NMGS" (31), NMGDH (2), and GMAS (21, 25, 37), the genes involved in these pathways have been studied only recently (24, 38).

Methylocella silvestris BL2 is a facultative methane-oxidizing bacterium belonging to the *Alphaproteobacteria* (11, 13, 35). It can grow on methylamine as a sole carbon and nitrogen source. Here we report the characterization of a gene cluster in *M. silvestris* BL2 involved in MMA metabolism and demonstrate that *gmas* is essential in MMA metabolism by this bacterium.

MATERIALS AND METHODS

Cultivation of *Methylocella silvestris*. Cells were grown at 25°C in a fed-batch mode in a 5-liter fermentor using diluted nitrate mineral (DNMS) medium, as

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TABLE 1. List of oligonucleotides used in this study

Target	Sequence (5′–3′)		Product size (bp)	Annealing temp (°C)
	Forward primer	Reverse primer		
Msil2632	GCGGCATAGTTGGATTGTTT	CATTCCAGCAAGATCGAACC	412	55
Msil2633	CTGCGAAAATCTTCGACCTC	GATCGAGCCTTTGACGAGAA	400	55
Msil2634	CTGAAAATTCCCGTCACCAT	TTCTCCCAATCGGTGATTTC	421	55
Msil2635	CAAAGAGTGTGGCGTCAAGA	TCTCGGTGATGACGTCGTAG	460	55
Msil2636	CGTGCTCAATCTCTGCCATA	GAATCCCTTGGTGGTTTCAA	327	55
Msil2638	GGCCGCAATATTTCCCTATT	TCGCAGATTGTGATCTCAGC	510	60
Msil2639	GACCGTGTTTGACGTCTCG	TCCGTCAACGCATGATAGAG	250	60
Msil2632/Msil2633	CCGATCAATATGTCGCCTTC	TGACGTCGCCTTTCACATAG	423	55
Msil2633/Msil2634	GCGCCTTGTATCATTTCCAC	GCGCTTGGAGATTTTCTGAC	598	55
Msil2634/Msil2635	CGCCTTGCAAACTACCTTTC	TATCAAGCCATGTCGCAAAG	411	55
Msil2635/Msil2636	CATCAGGAATGGCAGGATTT	CGAATTCCTTGGCGAGATAA	333	55
Msil2636/Msil2638	AATCGAAGGGCTGTTCTTCA	CGTGTCGCCGAAATATCC	591	60
Msil2638/Msil2639	CGCTGAACGATCGCAAAC	GAGCGACGCCAACAGCTC	547	60
Construction of *gmas* mutant	CCTGAGAATTCATGATCATGTCGGCATCC	CTGGAAGGATCCTCAGCAGTCGAGCGTCTGCTC	1,308	52
Amplification of *kan* gene cassette	GGTAGGTCGACGCATGCGAGCTCGGAAAGCCACGTTGTGTCTC	GGTAGGTCGACGCATGCGAGCTCAAGGTGTTGCTGACTCATAC	1,184	55
Confirmation of *gmas*::*kan* mutant	GCCTTGCAAACTACCTTTCG	CGAATTCCTTGGCGAGATAA	2,591 (for mutant), 1,665 (for wild type)	55

described by Theisen and colleagues (35). A total of 10 mM methylamine hydrochloride or 10 mM methanol plus 2 mM NH_4Cl was used as a carbon and nitrogen source. Cells were harvested at late exponential phase, resuspended in 10 mM 1,4-piperazinediethanesulfonic acid (PIPES) buffer (pH 7.6), and then stored at −80°C. To test whether sarcosine could be used as a substrate for *M. silvestris*, 10 mM sarcosine was used with or without 10 mM methanol.

To test if the *gmas* mutant could grow on MMA, a nitrogen-free DNMS medium was used. MMA was added at 5, 10, or 20 mM as the sole carbon and nitrogen source. A control, wild-type *M. silvestris*, was grown on either DNMS medium with methanol or nitrogen-free DNMS medium with 10 mM MMA. All growth experiments were set up in triplicate using 120-ml serum vials containing 20 ml medium, with an inoculum size of 5%. The serum vials were incubated at 25°C in a shaker (150 rpm).

HPLC-MS analyses of GMA. GMA was extracted from 100 μl frozen cells by vigorously vortexing them in 1 ml methanol for 1 min. Cells were then removed by centrifugation. The supernatant was heated at 60°C to remove methanol by evaporation. The residual powder was dissolved in 20 μl of water, and the solution was kept at −20°C prior to mass spectrometry (MS) analyses, which were carried out using an Agilent 1100 high-performance liquid chromatography (HPLC) system, with a diode array detector (DAD) coupled to a Bruker HCT-plus (high-capacity ion trap) mass spectrometer. The HPLC column was obtained from Agilent (Zorbax reverse-phase HPLC column Rx-C_{18}, 150 by 4.6 mm and particle size of 5 μm with C_{18} guard cartridge). Samples were filtered through a 0.2-μm microspin filter before injection. The mobile phases used were water with 0.1% trifluoroacetic acid (TFA) (A) and methanol with 0.1% TFA (B). Gradient settings used were 0 to 2 min at 100% A, 2 to 25 min from 100% A to 100% B, 25 to 30 min at 100% B, 30 to 35 min of equilibration to 100% A, and 35 to 45 min at 100% A. The flow rate was 1 ml min^{-1}, and 10% of the flow was diverted to MS. The DAD setting was 210 nm, 254 nm, and 280 nm. The MS setting was full scan, with electrospray ionization in positive mode, nebulizer gas at 10 liters min^{-1}, dry gas at 300°C and 40 lb/in^2, and capillary exit at 65 V. GMA was identified based on the elution time, and molecular mass was compared to synthesized standards, which were prepared and purified according to a previously published method (27).

Protein analyses. Cells were broken for protein analyses and enzyme assays by passing them three times through a French pressure cell (American Instrument Co.) at 110 MPa. Cell debris was removed by centrifugation at 6,000 × *g* for 15 min. Where necessary, soluble and membrane protein fractions were further separated by ultracentrifugation at 150,000 × *g* for 2 h, and the membrane fraction was washed once with buffer (10 mM PIPES, pH 7.6), followed by ultracentrifugation for 1 h under the same conditions. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad). One-dimensional protein analyses were carried out using a precast NuPage Bis-Tris gel (10%), according to the manufacturer's protocol (Invitrogen). Gels were stained with Coomassie brilliant blue R-250, and bands were excised, digested with trypsin, and analyzed using matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS) and tandem mass spectrometry at the Biological Mass Spectrometry and Proteomics Facility, Department of Biological Sciences, University of Warwick, as described previously (32).

RNA extraction and reverse transcription (RT)-PCR. RNA was extracted from frozen cells using either an RNeasy mini kit (Qiagen) or the method described by Gilbert and colleagues (14). Trace DNA contaminants in the RNA were further removed by DNase I (Qiagen) digestion, and RNA was then recovered using the RNeasy minikit (Qiagen). Reverse transcription was performed using the SuperScript II system (Invitrogen). Reverse transcription and PCR were carried out using a Bio-Rad thermocycler. Primers used are listed in Table 1.

Enzyme assays. All enzyme assays were carried out in triplicate using cell-free crude extract in 10 mM PIPES buffer (pH 7.6) at room temperature (22°C), unless otherwise stated. GMA synthetase (MMA + glutamate + ATP → ADP + P_i + GMA) activity was measured by quantifying the production of γ-glutamylhydroxamate from glutamate and hydroxylamine in the presence of ATP and Mg^{2+} by monitoring the change in absorbance at 540 nm. The assay was originally developed to quantify the ability of glutamine synthetase to form glutamine (4) but later adapted to quantify GMAS activity (24, 37). The following concentrations of substrates were used: glutamate (50 mM), hydroxylamine hydrochloride (15 mM), $MgCl_2$ (20 mM), and ATP (5 mM).

NMGDH activity was measured using 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron acceptor with or without KCN (1 mM), as described by Bamforth and Large (2). The initial rate of decrease in A_{600} was recorded continuously for 5 min (within the linear phase). MMA dehydrogenase was assayed in the same way, except that methylamine hydrochloride (final concentration, 10 mM) was used as the substrate to initiate the reaction. In addition, NMGDH activity was also measured by quantifying NMG-dependent formaldehyde production using the method described below.

The activity of GMA dissimilation enzymes was measured using the following two methods: (i) GMA-dependent formaldehyde production and (ii) GMA-dependent ammonium production. Formaldehyde concentration was determined colorimetrically using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald reagent; Sigma). The reaction was started by adding GMA, and absorbance at 550 nm was recorded before and after incubation for 10 min to measure the initial rate of conversion within the linear phase (12). Prior to assaying ammonium, the cell-free enzyme extract was dialyzed to remove ammonium and MMA from the culture using a Slide-A-Lyzer 3.5-kDa-molecular-mass-cutoff dialysis cassette (Thermo Scientific), dialyzing for 2 h against 50 mM PIPES buffer (1 liter, pH 7.6) at 4°C. GMA-dependent ammonium released before and after 30 min of incubation was determined colorimetrically using Nessler's reagent (Sigma), measuring the change of absorbance at 440 nm (7). A decrease in absorbance was observed when α-ketoglutarate was present in the assay. This

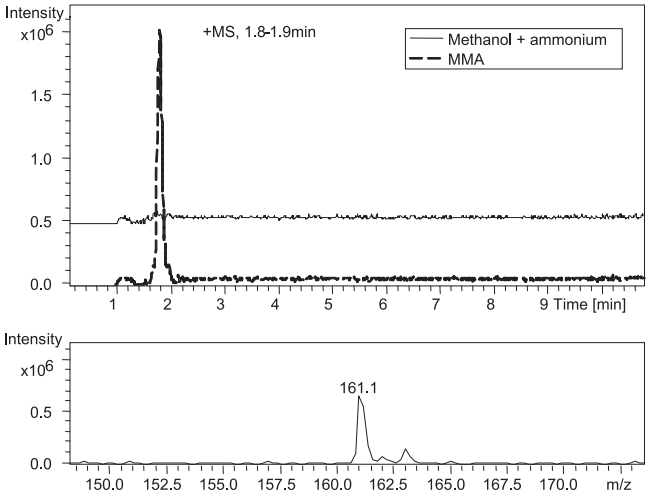

FIG. 1. (Top) Comparison of extracted ion chromatograms (EIC; *m/z* 161) of methanol extract of *Methylocella silvestris* cells grown on methylamine (dashed line) and methanol plus ammonium (solid line). (Bottom) The peak identified with a mass-to-charge ratio of 161.1 is γ-glutamylmethylamide.

was due to the glutamine synthetase/glutamate synthase activity in the presence of residual ammonium after dialysis (data not shown).

"NMGS" activity was measured indirectly by quantifying glutamate and MMA-dependent formaldehyde production as described previously (24). The assay was initiated by adding either glutamate (final concentration, 10 mM) or MMA (final concentration, 10 mM).

Bioinformatics. Sequence similarity searches were performed with GenBank using the BLASTP program against the nonredundant protein sequence database and the Swiss-Prot protein sequence database. Protein sequences of type I, type II, and type III glutamine synthetases were downloaded from GenBank and aligned using the ClustalX program (36). Phylogenetic analysis was performed using the MEGA4 program (34).

Marker exchange mutagenesis of *gmas*. To construct a *gmas* mutant of *M. silvestris*, the *gmas* gene was amplified by PCR and inserted into pUC19 under the EcoRI and BamHI sites. The 258-bp SacI fragment from the *gmas* gene was then removed and replaced with a kanamycin gene cassette, which was amplified from the plasmid pCM184 by PCR using the primers listed in Table 1 (30). The plasmid was cut with EcoRI and BamHI, and the 2.2-kb fragment containing the kanamycin gene cassette was then gel purified and electroporated into *M. silvestris* using the method of Kim and Wood (19), with minor modifications. A total of 500 ng of DNA was added to 100 μl of cells. The electroporation settings used were resistance at 400 Ω, voltage at 2.2 kv, and a cuvette of 1 mm. Cells were recovered at 25°C overnight with DNMS medium containing methanol (10 mM) before plating. Potential mutants were selected on DNMS (20 μg ml^{-1} Kan) agar plates, with methanol (10 mM) as the sole carbon source. Mutation of the *gmas* gene was confirmed by diagnostic PCR and subsequent DNA sequencing.

RESULTS

MMA metabolism by *Methylocella silvestris* involves GMA. *M. silvestris* can utilize MMA as the sole carbon and nitrogen source (13). Its genome is available in GenBank (accession number CP001280), and the genome does not contain any candidate coding sequences for MMA oxidase or MMA dehydrogenase. In addition, dye-linked MMA dehydrogenase assays were performed, and no activity was found using cell extract from cells grown on MMA as a sole carbon and nitrogen source.

Experiments were then carried out to investigate if *M. silvestris* used the methylated amino acid pathway for MMA metabolism. HPLC-MS analysis showed that when *M. silvestris* was grown on MMA, GMA could be detected, whereas no GMA was found when *M. silvestris* was grown on methanol (Fig. 1). No NMG was detected in either MMA-grown cultures or methanol-grown cultures by HPLC-MS analysis. Assays were then carried out to determine activities of key enzymes involved in the GMA- and NMG-mediated metabolic pathways (Table 2). The γ-glutamylhydroxamate assay showed that GMAS/glutamine synthetase activities were approximately 3-fold higher in MMA-grown cultures than in methanol-grown cultures. The GMAS/glutamine synthetase activity detected by the γ-glutamylhydroxamate assay in methanol-grown cultures was due to glutamine synthetase rather than GMAS, since GMAS is not expressed under these conditions (see below). Furthermore, assays for NMGDH were carried out using two methods. Dye-linked NMGDH activity was found only when the bacterium was grown on MMA and not when it was grown on methanol plus ammonium. This activity was relatively low (4.4 ± 0.6 nmol min^{-1} mg protein^{-1}). Dye-linked NMGDH activity was approximately 4-fold lower without KCN (final concentration, 1 mM), added in order to inhibit cytochrome oxidases, as suggested by Bamforth and Large (2). A more robust assay for NMGDH was used to quantify the production of formaldehyde from NMG. This activity was, again, detectable only in MMA-grown cultures but was higher (16.6 ± 0.7 nmol min^{-1} mg protein^{-1}) than that obtained using the dye-linked NMGDH assay, indicating that DCPIP was a poor artificial electron acceptor for this enzyme. Assays using cytochrome *c* from horse heart did not yield any activity for NMGDH from *M. silvestris*. In addition, no activity of "NMGS" was detected in either MMA-grown or methanol-grown cultures using the method described by Latypova and colleagues (24).

Identification and characterization of a gene cluster involved in MMA metabolism. A proteomic analysis was carried out to investigate the proteins involved in MMA metabolism by *M. silvestris*. By comparing polypeptide profiles of cells grown on methanol or MMA, it was obvious that several proteins were highly induced by MMA in both the soluble fraction and membrane fraction of MMA-grown cells (Fig. 2A). These polypeptides were cut from the gel and sequenced by tandem mass spectrometry. The results indicated that proteins encoded by a gene cluster were highly induced in MMA-grown

TABLE 2. Key enzyme activities in cells grown on MMA or methanol

Assay	Activity (nmol min^{-1} mg protein^{-1}) in cells grown on[a]:	
	MMA	Methanol plus ammonium
GMAS	31.1 ± 0.4	11.9 ± 0.9
NMGDH		
DCPIP	4.4 ± 0.6	ND
Formaldehyde	16.0 ± 0.7	ND
GMA dissimilation activity		
Formaldehyde	4.74 ± 0.12	ND
Ammonium	7.98 ± 0.49	ND

[a] Values are means ± standard deviations from three assays. ND, not detectable.

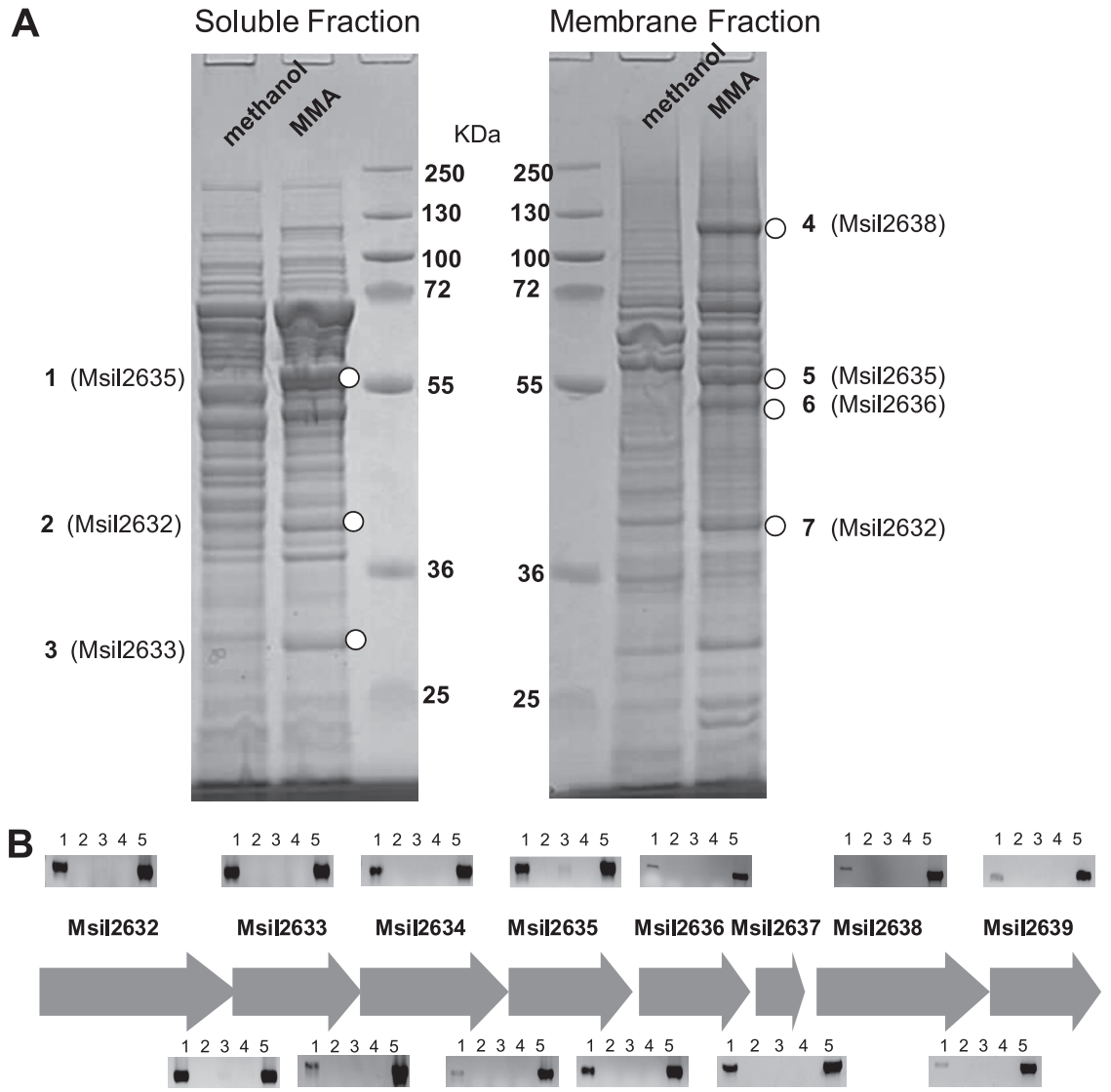

FIG. 2. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of soluble and membrane proteins from cells grown on MMA alone and methanol plus ammonium. Bands highlighted (1 to 7) were cut from the gel and analyzed by matrix-assisted laser desorption ionization–mass spectrometry and tandem mass spectrometry. (B) Agarose gel photos of RT-PCR products showing the eight-gene cluster, which was induced by MMA. Agarose gel photos above each gene show the induction of each gene by MMA only, and gel photos above intergenic regions show that this gene cluster is cotranscribed. RT-PCR products using mRNA extracted from MMA-grown cells and methanol-grown cells are shown in lanes 1 and 3, respectively. Corresponding controls without reverse transcriptase are shown in lanes 2 and 4, respectively. Lane 5 is the positive control for PCR using genomic DNA from *Methylocella silvestris*.

cells and were, therefore, likely to be involved in MMA metabolism by *M. silvestris* (Table 3; Fig. 2A).

The results from BLAST searches for each gene in this cluster are shown in Table 4. The first three open reading frames (ORFs; Msil2632 to Msil2634) encode polypeptides with high similarities to individual domains of the glutamate synthase large subunit, containing a glutamine amidotransferase domain, a GXGXG motif, and a highly conserved flavin mononucleotide (FMN)-binding domain, respectively. All the BLASTP hits matched with significant identities are putative genes with no known experimentally validated functions. ORF Msil2635 encodes a glutamine synthetase-like protein; however, sequence analysis indicated that this gene

TABLE 3. Identification of polypeptides induced by MMA

Band	ORF identification	Calculated molecular mass from protein sequence (kDa)	No. of polypeptides detected	Sequence coverage (%)
1	Msil2635	48.4	5	21
2	Msil2632	32.0	5	21
3	Msil2633	24.7	4	24
4	Msil2638	104.3	7	10
5	Msil2635	48.4	8	17
6	Msil2636	44.8	6	18
7	Msil2632	32.0	4	20

TABLE 4. Gene description and BLAST search results

ORF	Length (aa)	Top BLASTP match (identity [%])	Gene assignment[a]		Note
			a	b	
Msil2632	299	Glutamine amidotransferase, class II (*Azorhizobium caulinodans* ORS 571 [67]) Glutamine amidotransferase, class II (*Xanthobacter autotrophicus* Py2 [65])	*gltB1*	*mgsA*	Contains a GlxB-like glutamine amidotransferase domain
Msil2633	235	Glutamate synthase domain 3-like protein (*Methylobacterium populi* BJ001 [72]) Glutamate synthase family protein (*Xanthobacter autotrophicus* Py2 [66])	*gltB3*	*mgsB*	Contains a GXGXG motif commonly found in glutamate synthase
Msil2634	444	Putative glutamate synthase GltB2 subunit (*Methylobacillus flagellatus* KT [87]) Glutamate synthase domain 2-like protein (*Azorhizobium caulinodans* ORS 571 [86])	*gltB2*	*mgsC*	Contains a highly conserved FMN-binding domain in GltS
Msil2635	435	Putative type III glutamine synthetase (*Rhodopseudomonas palustris* HaA2 [69]) γ-Glutamylmethylamide synthetase (*Methylovorus mays* [41])	*gltIII*	*gmas*	Does not contain key residues for ammonium binding
Msil2636	416	Putative sarcosine oxidase β subunit (*Azorhizobium caulinodans* ORS 571 [75]) Sarcosine oxidase β subunit (*Corynebacterium* sp. P-1 [49])	*soxB*	*mgdA*	
Msil2637	95	Putative sarcosine oxidase, δ subunit (*Bradyrhizobium* sp. BTAi1 [55]) Sarcosine oxidase δ subunit (*Corynebacterium* sp. P-1 [37])	*soxD*	*mgdB*	
Msil2638	984	Putative sarcosine oxidase α subunit (*Methylobacterium populi* BJ001 [55]) Sarcosine oxidase α subunit (*Corynebacterium* sp. P-1 [36])	*soxA*	*mgdC*	Contains two domains of the glycine cleavage T-protein
Msil2639	209	Putative sarcosine oxidase γ subunit (*Rhodopseudomonas palustris* HaA2 [37]) Sarcosine oxidase γ subunit (*Corynebacterium* sp. P-1 [31])	*soxG*	*mgdD*	

[a] a, assigned based on BLASTP matches; b, assigned based on the similarity between Msil2632 to Msil2639 and the gene cluster of *Methyloversatilis universalis* (24).

is lacking a conserved ammonia-binding site (D^{50}, S^{53}, and Y^{179}), which is commonly found in glutamine synthetases (26). This gene also shows 41% identity to a newly characterized GMAS from *Methylovorus mays* (38) (Fig. 3). In both Msil2635 and GMAS from *Methylovorus mays*, the three key ammonium-binding residues are nonpolar rather than polar ($D^{50} \rightarrow G^{50}$, $S^{53} \rightarrow A^{53}$, and $Y^{179} \rightarrow C^{179}$). The last four ORFs in this gene cluster showed 35 to 75% sequence identities to putative heterotetrameric sarcosine oxidase subunits. Msil2636 and Msil2638 had 36% and 49% identity, respectively, to characterized sarcosine oxidase subunits of *Corynebacterium* sp. P-1 (10). However, when tested, *M. silvestris* failed to grow on sarcosine supplied as either a sole carbon source or a sole nitrogen source.

To further investigate whether this gene cluster is induced by MMA, transcriptional analyses were performed with RNA extracted from MMA- and methanol-grown cultures. The results (Fig. 2B) indicated that the genes in this cluster were induced by MMA, whereas no transcripts could be detected in methanol-grown cells. Furthermore, RT-PCR targeting the intergenic region yielded products of the expected sizes, indicating that the genes were cotranscribed as a single operon.

The activity of the GMA-dissimilating enzyme is dependent on glutamate. The fate of GMA in microorganisms which use the methylated amine pathway for MMA metabolism is controversial. We therefore decided to further explore whether GMA could be dissimilated by *M. silvestris*. The results are shown in Fig. 4. Formaldehyde production was seen when GMA was incubated with cell-free crude extract, and this was stimulated by glutamate. Ammonium production from GMA was also observed. This was also dependent on glutamate. These experiments indicated that GMA, via the presence of glutamate and release of ammonium, may be transformed into NMG, which is further oxidized by NMG dehydrogenase to yield formaldehyde.

A *gmas* mutant cannot grow on MMA as either a sole carbon source or a sole nitrogen source. To investigate if the putative *gmas* gene, which was induced by MMA, is essential in MMA metabolism by *M. silvestris*, we constructed a mutant by marker exchange. This mutant can grow on methanol in the presence of ammonium but failed to grow on MMA as a sole carbon and nitrogen source at 5, 10, or 20 mM (Fig. 5). In addition, unlike wild-type *M. silvestris*, this mutant cannot grow on MMA as a sole nitrogen source in the presence of methanol using nitrogen-free DNMS medium (data not shown).

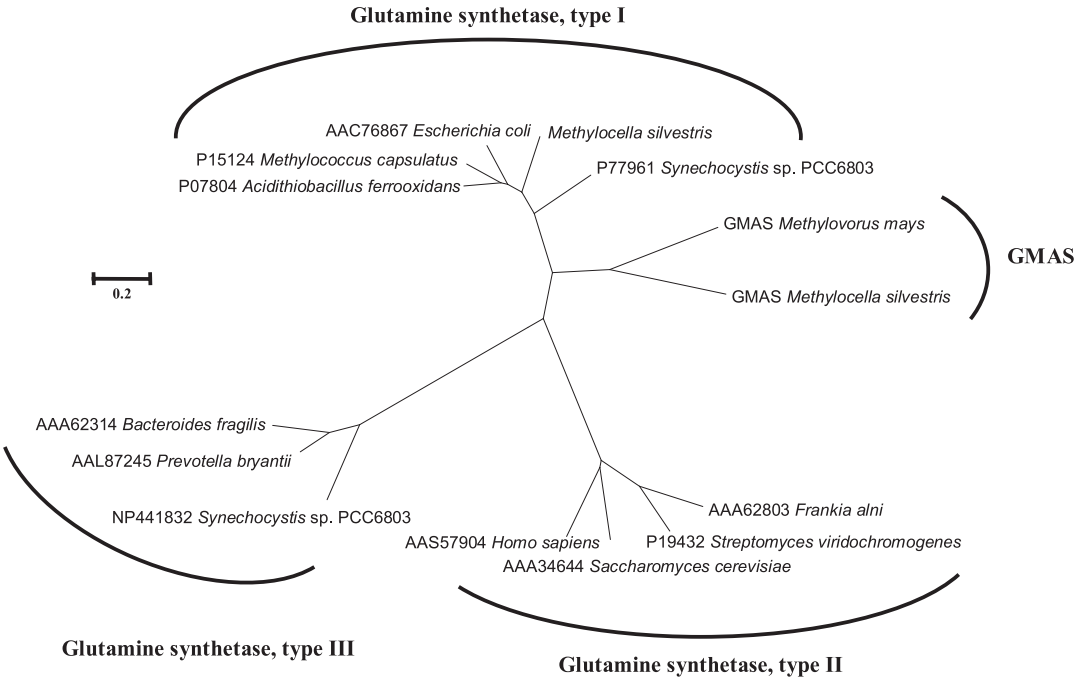

FIG. 3. An unrooted phylogenetic tree showing the relationship between γ-glutamylmethylamide synthetases (GMAS) and three types of glutamine synthetases. Amino acid sequences were aligned using the ClustalX program, and the tree was constructed using MEGA4 (~350 amino acids [aa] for γ-glutamylmethylamide synthetases, ~370 aa for type I glutamine synthetases, ~335 aa for type II glutamine synthetases, and ~440 aa for type III glutamine synthetases). The scale bar represents 2 substitutions per 10 amino acids.

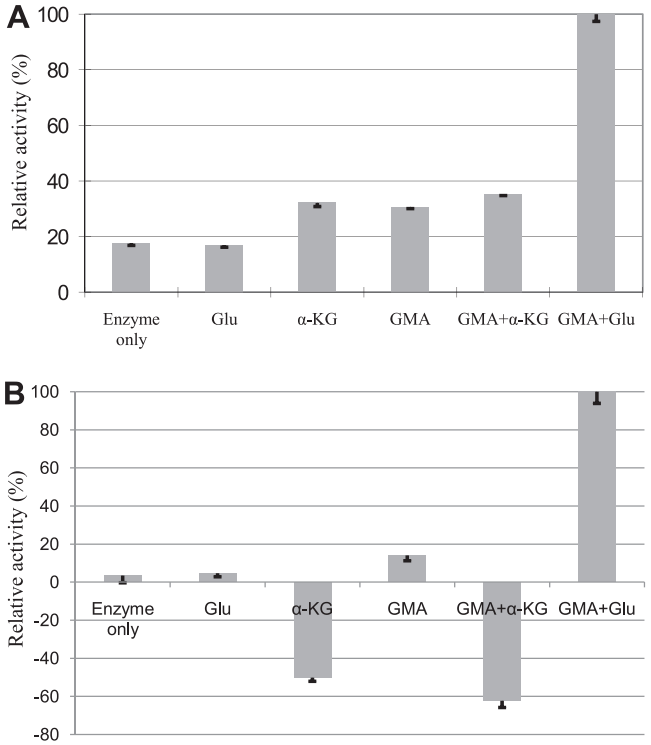

FIG. 4. GMA-dissimilating enzyme activities determined by quantifying GMA-dependent formaldehyde production (A) or ammonium production (B). The activities with GMA and Glu were chosen as 100%, which were 4.74 ± 0.12 nmol min^{-1} mg $protein^{-1}$ for the formaldehyde assay and 7.98 ± 0.49 nmol min^{-1} mg $protein^{-1}$ for the ammonium assay. Glu, glutamate; α-KG, α-ketoglutarate; GMA, γ-glutamylmethylamide. Means and standard deviations of the results from three replicates are shown.

DISCUSSION

Through comparative proteomic analyses, we revealed that a cotranscribed cluster of eight genes is involved in MMA metabolism by *M. silvestris*. Recently, a similar gene cluster was identified in *Methyloversatilis universalis* and was designated NMGS (*mgsABC*), GMAS, and NMGDH (*mgdABCD*), respectively (24). No attempt was made to elucidate the functions of putative *mgsABC* (Msil2632 to Msil2634) and *mgdABCD* (Msil2636 to Msil2639) identified in *M. silvestris* in this study; however, it is likely that they are NMGS and NMGDH, respectively.

GMA, which is induced by MMA, was found as an early product in the metabolism of MMA by *Aminobacter aminovorans* strain MS (23), suggesting a key role for GMA in MMA metabolism by this bacterium. It was also found that the enzyme, which was responsible for GMA synthesis, was similar to, but distinct from, glutamine synthetase. GMAS and glutamine synthetase from *Aminobacter aminovorans* strain MS were subsequently purified, and it was demonstrated that GMAS was more specific for GMA (25). In *M. silvestris*, a separate bona fide type I glutamine synthetase gene is present in the genome, and this enzyme is expressed in both methanol- and methylamine-grown cells (data not shown). The *gmas* homolog (Msil2635), which is induced by MMA (Fig. 2B) in *M. silvestris*, is likely to be GMAS. The translated amino acid of this protein showed high sequence identity to a characterized GMAS from *Methylovorus mays* (38), and phylogenetic analyses showed that these two proteins, which are related to type I glutamine synthetases, form a separate branch (Fig. 3). Moreover, similar to GMAS from *Methylovorus mays*, GMAS from

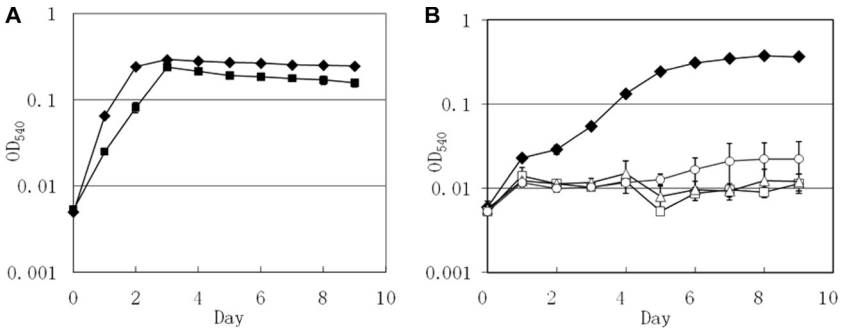

FIG. 5. (A) Growth curves of the wild type (filled diamonds) and the *gmas*::*kan* mutant (filled squares) of *Methylocella silvestris* grown on methanol (10 mM) with ammonium (2 mM). (B) Growth curves of the wild type (filled diamonds, 10 mM) and the *gmas*::*kan* mutant (open triangles, 5 mM; open squares, 10 mM; open circles, 20 mM) of *Methylocella silvestris* grown on MMA alone at different concentrations. Means and standard deviations of the results from three replicates are shown. OD_{540}, optical density at 540 nm.

M. silvestris lacks key residues that are crucial in the binding of ammonium, suggesting that these enzymes do not prefer ammonium and thus are likely to be GMAS rather than glutamine synthetase.

The fate of GMA in bacterial MMA metabolism is controversial. Latypova and colleagues found that GMA was a key intermediate in *Methyloversatilis universalis* when it was grown on MMA, but no GMA-dissimilating enzyme activity could be detected (24). Different observations were made in other studies. For example, using ^{14}C-labeled GMA, Loginova and colleagues demonstrated that GMA was converted to NMG in *Hyphomicrobium vulgare* and that the reaction was dependent on glutamate (29). GMA dissimilation activity was also found in a *Methylophaga* strain, and the reaction was dependent on α-ketoglutarate and ammonium (20, 22). In *M. silvestris*, we were unable to show a direct conversion of GMA to NMG (data not shown); however, we demonstrated that there is glutamate-dependent, but not α-ketoglutarate-dependent, GMA-dissimilating enzyme activity which releases formaldehyde and ammonium from GMA (Fig. 4). This is similar to what has been found in *Hyphomicrobium vulgare*, where the conversion of GMA to NMG was also dependent on the presence of glutamate. The demonstration of α-ketoglutarate- plus ammonium-dependent, but not glutamate-dependent, GMA dissimilation enzyme activity in *Methylophaga* spp. is probably misleading, since the assay was carried out in a buffer containing 100 mM ammonium (20), which might have inhibited the glutamate-dependent GMA dissimilation activity. In fact, high glutamine synthetase/glutamate synthase activity was found in this bacterium when it was grown on MMA (20), and α-ketoglutarate and ammonium may have been converted to glutamate, which might have served as a true substrate for the GMA-dissimilating enzyme in this *Methylophaga* strain. GMAS in *M. silvestris* seems to be essential, and mutation of the corresponding gene (*gmas* homologue, Msil2635) resulted in a mutant which failed to grow on MMA as either a sole carbon source or a sole nitrogen source (Fig. 4B).

We therefore propose the pathway of MMA metabolism by *M. silvestris* shown in Fig. 6, in which MMA is metabolized, via GMA and NMG, to formaldehyde and ammonium. Formaldehyde produced is either further oxidized to CO_2 to generate energy and reducing equivalents or assimilated into cell biomass, probably through the serine cycle. Ammonium produced in this pathway is used as a nitrogen source which is probably assimilated through bona fide glutamine synthetase and glutamate synthase (genes encoding glutamate dehydrogenase or alanine dehydrogenase are not present in the genome). However, we were unable to detect NMG from MMA-grown cells of *M. silvestris*. This is probably due to the fact that accumulation of GMA or NMG is related to growth state, as demonstrated previously (16a). Although it was suggested that GMA-dependent and NMG-dependent MMA metabolism by bacteria involved two different pathways (1, 24), our data and those of others suggest that it is more likely that these two may indeed be the same pathway (8, 29). It has been shown that glutamate-dependent ammonium and formaldehyde production from GMA also occurs in *Aminobacter aminovorans* strain MA when it is grown on MMA (8). This strain has been studied extensively, and it uses the NMG-mediated pathway for MMA metabolism (15, 31, 33). The NMG-mediated pathway for MMA metabolism was proposed, based mainly on studies using this strain. The key lies in whether GMA or MMA is the true substrate for this so-called "NMG synthase" ("NMGS"). The purified "NMGS" from strain MA is specific for glutamate but not for MMA, and indeed, a number of amines can substitute for MMA (31). There is, therefore, an urgent need to purify "NGMS" and to reexamine the specificity of this en-

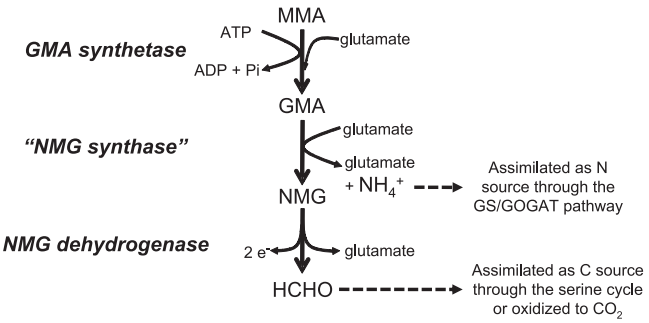

FIG. 6. Proposed pathway of MMA metabolism by *Methylocella silvestris*. Formaldehyde produced from this pathway is either assimilated as a carbon source through the serine cycle or further oxidized to CO_2, and the ammonium produced is assimilated as a nitrogen source through the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway. e^-, electron.

zyme. Kinetic studies are also required to finally resolve the controversial roles of the GMA- and NMG-dependent pathways for MMA metabolism by bacteria.

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